Signaling and Communication in Plants

Markus Geisler *Editor*

Plant ABC Transporters

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Plant ABC Transporters

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Preface

ABC (ATP-binding cassette) proteins are ubiquitous, membrane-intrinsic transporters that catalyze the primary (ATP-dependent) movement of their substrates over biological membranes. Until know, the special challenge of ABC transporter work lies in the identification of ABC transporter substrates. Therefore, in higher plants, ABC transporters were—in functional analogy to their mammalian orthologs—initially identified as central part of a detoxification process in that they sequester conjugated xenobiotics from the cytoplasm into the central vacuole. However, mainly genetic work in the last decade has provided the discovery of unexpectedly diverse ABC transporter substrates that include beside chlorophyll catabolites and xenobiotic conjugates, heavy metals, lipids, terpenoids, lignols, and organic acids. According to their associated wide biological function, plant ABCs were found beside on vacuolar membranes also on the plasma membrane and on membranes of other organelles, including the plastids, peroxisomes, mitochondria and the endoplasmic reticulum. The discovery that members of the ABCB and ABCG family are involved in the cellular and intercellular movement of phytohormones, such as auxins (IAA and IBA), abscisic acid (ABA), cytokinin, and most probably also strigolactones, has boosted their investigation and provided a new perception of the whole family. Further, recent work on ABC transporters has left the Arabidopsis limits and been extended to dicot and monocot crop plants, which has caused considerable interest also outside the plant community.

This book is therefore devoted to the exciting plethora of plant ABC transporter substrates and highlights their tightly connected biological functions that accordingly reach from cellular detoxification, over development, to symbiosis and defense. It contains a special focus on "phytohormone transporters" and several chapters converging on the trafficking, regulation, and structure–function of ABCB-type auxin transporters. Moreover, it especially emphasizes the role of ABC transporters in plant defense and the symbiosis between plant and microorganisms, such as arbuscular mycorrhiza and rhizobia root nodules. Finally, it encompasses also a set of chapters that center on ABC protein structure and ABC evolution.

I would like to express my deepest gratitude to the ABC transporter community for pushing this exciting field so tremendously forward in the last decade. But also for contributing to this little milestone, making it what it was initially meant to be: a complete, timely, and beautiful overview on *Plant ABC Transporters* for the expert. And a teaser for the beginner: there is much more to explore!

Fribourg, Switzerland Markus Geisler
March 17, 2014

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ABC Transporters and Heavy Metals

Won-Yong Song, Jiyoung Park, Cornelia Eisenach, Masayoshi Maeshima, Youngsook Lee, and Enrico Martinoia

Abstract The first evidence showing that ABC transporters are involved in heavy metal resistance in eukaryotic cells has been obtained from experiments in *Schizosaccharomyces pombe* and *Saccharomyces cerevisae*, where a half-size transporter of the ABCB subclass and an ABCC-type transporter, respectively, have been shown to confer heavy metal tolerance. Biochemical studies have indicated that vacuolar ABC transporters should also play an important role in heavy metal detoxification in plants. But it was only recently that two ABCC-type transporters, AtABCC1 and AtABCC2, have been identified as major apo-phytochelatin and phytochelatin-heavy metal(oid) complex transporters. Several plasma membrane transporters have also been shown to confer heavy metal resistance. However, with the exception of STAR1, an UDP glucose exporter, which—by altering cell wall composition—confers aluminum tolerance, the substrates required to be transported to confer heavy metal resistance by these plasma membrane-localized ABC proteins are still not elucidated. A mitochondrial ABC transporter AtATM3 was shown to be required for plant growth and development.

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The different studies indicate that this transporter is important for the production of cytosolic iron sulfur complexes and molybdenum cofactors, prosthetic groups required for several enzymes. However, the final proof as to which substrate is transported by AtATM3 is still missing. Several laboratories took advantage of the fact that ABC transporters are involved in heavy metal tolerance to generate transgenic plants suitable for phytoremediation. The results show that overexpression of ABC proteins alone is not sufficient to produce plants that can efficiently decontaminate soils, but they indicate that this class of transporters, when combined with other transporters and enzymes involved in heavy metal transport and detoxification, may prove a good solution to produce plants that can stabilize, and in the long term clean up, soils contaminated with heavy metals.

1 Introduction

All living cells require heavy metals such as Fe, Zn, Mn, Cu, Ni, or Mo as cofactors for enzymes and transcription factors. As other organisms, plants have to tightly regulate uptake, allocation, and storage of these essential heavy metals in order to allow for optimal growth. On the one side, a deficiency in these metals causes growth retardation and developmental defects throughout a plant's entire life cycle; on the other side, the uptake of excess heavy metals would lead to toxicity syndromes.

Non-essential, toxic heavy metals are present in natural soils, and volcanic eruptions are often accompanied by the release of toxic heavy metals. During the industrialization period of the late nineteenth and early twentieth century anthropogenic release of heavy metals has increased dramatically. Mining, waste incinerators, pesticides, and fertilizers contaminated our environment. Furthermore, in Bangladesh and India water wells initially built to supply clean water to the population were later found to contain toxic amounts of arsenic, which led to a contamination of rice paddy fields (Zhao et al. 2010; Meharg and Rahman 2003; Williams et al. 2005).

Non-essential heavy metals may hijack transporters required for the uptake of essential heavy metals, because of the two metal species' chemical similarity, and consequently enter and accumulate in the plant cell. It has been shown that the iron/zinc transporter IRT1 is the major entry point for toxic cadmium (Vert et al. 2002), while high-affinity phosphate transporters and members of the MIP family import arsenate(V) and arsenite (III), respectively (Zhao et al. 2010).

A multitude of transporters belonging to different classes are involved in uptake, distribution and sequestration of metal ions. Uptake of heavy metals occurs mainly by cation channels and symporters, such as members of the ZRT/IRT1 protein family, which are responsible for the uptake of iron and zinc or the copper transporter COPT1 (Palmer and Guerinot 2009). P-type ATPases have been

shown to be required for the export of zinc into the xylem and its translocation to the shoot (Hussain et al. 2004; Verret et al. 2004). Alternatively, antiporters may also release heavy metals into the xylem, as has been suggested for the putative iron exporter ferroportin (Morrissey et al. 2009). P-type ATPases have also been demonstrated to play an important role in transporting copper from the cytosol into the chloroplast stroma and from the stroma into the thylakoid lumen (Shikanai et al. 2003; Burkhead et al. 2009). Storage and concomitant detoxification of excess heavy metals generally occurs within the vacuole (Martinoia et al. 2007). The last decade saw the identification of a large number of vacuolar heavy metal transporters that import excess heavy metals but that also export them, if the plant metabolism requires the supply of a specific heavy metal (Martinoia et al. 2007, 2012). In order to further reduce the toxicity of heavy metals, plants produce chelating agents, mainly carboxylates and the glutathione-derived phytochelatins (Grill et al. 1989; Clemens 2006), which are also transported into the vacuole.

In contrast to bacteria, to date no plant ABC transporter has been shown to be involved in essential heavy metal uptake or release at the plasma membrane, while many bacterial ABC transporters were reported for essential metal ion transport (Self et al. 2003; Napolitano et al. 2012). Instead, many plant ABC transporters identified up to now are involved in toxic metal transport and thus protect plants from the harmful effects of toxic heavy metals. It will be worthwhile to investigate why higher organisms have evolved ABC transporters that are mainly exporters, whereas bacteria evolved to possess a higher numbers of nutrient importers.

2 The Complex Functions of HMTs/ATMs

Plants, fungi and some yeast are known to produce glutathione-derived heavy metal chelators, phytochelatins, in response to the presence of cadmium, lead, arsenic, and some other heavy metals (Grill et al. 1989; Cobbett 2000). The first putative vacuolar phytochelatine transporter was identified in *Schizosaccharomyces pombe* and named SpHMT1. SpHMT1 is a half size ABC transporter of the ABCB subclass. The corresponding mutant, *hmt1*, was hypersensitive to cadmium and the authors could link the sensitivity to a strongly reduced amount of high-molecular weight phytochelatin–cadmium (HMWPC-Cd-S-2) complexes (Ortiz et al. 1992). Transport assays with vesicles isolated either from *hmt1 S. pombe* or from *S. pombe* form hmt1 mutant having empty vector SpHMT1 revealed that HMT1 transports apo-phytochelatins as well a phytochelatin–cadmium complexes, but not Cd^{2+}, GSH, GSSG, or glutathione conjugates (Ortiz et al. 1995). Intriguingly, and in contrast to the phytochelatine biosynthesis mutant, the *hmt1* mutant was not sensitive to As or Hg. In order to see, whether cadmium resistance mediated by SpHMT1 required a transport process, they produced a catalytic SpHMT1 mutant not able to bind ATP. This mutated form of SpHMT1 did not confer cadmium resistance anymore even in the presence of phytochelatins.

The discovery that *Caenorhabditis elegans* produces phytochelatins, as do plants and some fungi, led to the discovery of a HMT1 homologue, CeHMT1, in this organism and generated the corresponding RNAi lines (Vatamaniuk et al. 2005). As observed for the *S. pombe hmt1* mutant, *Caenorhabditis elegans* was hypersensitive to cadmium when CeHMT1 was absent. Surprisingly, the worms carrying the RNAi construct were even more susceptible to cadmium than those mutated in phytochelatin synthase (PCS). In a subsequent report, Schwartz et al. (2010) showed that in contrast to SpPHMT1, CeHMT1 could confer heavy metal resistance not only to cadmium but also to As and Cu, albeit in a phytochelatin-independent manner. Interestingly, CeHMT1 and PCS are co-expressed in highly endocytic cells, called coelomocytes, suggesting that these cells play a central role in heavy metal detoxification in *C. elegans*. The observations that HMTs conferred cadmium resistance, but were unable to confer tolerance against As and Hg—both of which are known to form strong conjugates with phytochelatins—inspired two laboratories to re-evaluate the role of HMTs. Preveral et al. (2009) observed that SpHMT1 confers heavy metal resistance in *Saccharomyces cerevisiae* as well as in *E. coli*, both of which do not produce phytochelatins. However, SpHMT1 required glutathione to confer cadmium resistance. These results indicate that SpHMT1-dependent heavy metal resistance requires glutathione but not phytochelatins. Furthermore, Sooksa-nguan et al. (2009) showed that a HMT1 homologue from *Drosophila*, *DmHMT1,* which does not produce phytochelatins, was targeted to the vacuolar membrane in *S. pombe* and could rescue cadmium sensitivity in the *sphmt1* mutant. However, no phytochelatin transport activity could be observed in *S. pombe expressing DmHMT1*. In addition, the authors re-addressed the question of phytochelatin transport for SpHMT1 and could observe a slight but consistent decrease in vacuolar PC_2 content, but not for longer chain PCs. Consequently, Sooksa-nguan et al. (2009) suggested that SpHMT1 and PCS do not act in a direct, linear way and that SpHMT1 may contribute in a minor way to phytochelatin transport, while acting mainly in a way that is cadmium specific.

To date, HMT1 homologues that might reside in the vacuolar membrane of plants have not been identified, even though plants do encode for HMT1 homologues. Instead, in *Arabidopsis* (Chen et al. 2007; Rea 2007) as well as in *Chlamydomonas* (Hanikenne et al. 2005), the HMT1 homologues reside in mitochondria. Indeed, Atm1p, the HMT1 homolog in *S. cerevisiae*, was reported to reside in the inner mitochondrial membrane, and its transport activity was predicted to occur from the mitochondrial matrix to both, the intermembrane space as well as the cytosol (Leighton and Schatz 1995). Based on the observation that *atm1p* mutants accumulate iron within mitochondria, Atm1p has been suggested to function as a mitochondrial exporter for iron–sulfur clusters. Deletion of AtATM3/AtABCB25—one out of three Atm1p/HMT1 homologues of Arabidopsis—has a dramatic effect, causing dwarfism and chlorosis (Fig. 2a; Kushnir et al. 2001). The observation that AtATM3/AtABCB25 complements the yeast *atm1p* mutant indicates that both genes exhibit a similar function (Kushnir et al. 2001; Chen et al. 2007). In a later, detailed work, Bernard et al. (2009) provided further evidence that out of the three ATM *Arabidopsis* homologues, only ATM3 was

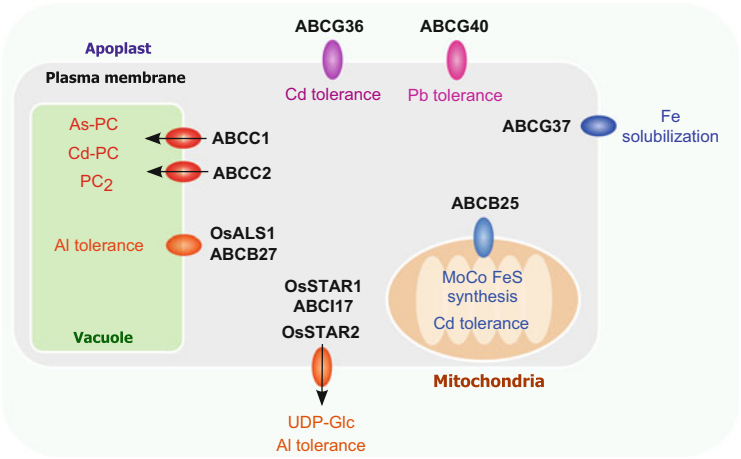

Fig. 1 Plant ABC transporters involved in heavy metal tolerance ABC transporters that have been reported to transport heavy metal(loid)s (marked with *arrows*) or involved heavy metal tolerance. Indicated transport proteins are from *Arabidopsis thaliana* unless specified otherwise

important for plant growth. Using a set of *atatm3* mutants, the group aimed to obtain information as to which substrate might be transported by AtATM3. Investigating enzymes containing Fe–S and molybdenum cofactors (MoCo) as prosthetic groups, they concluded that AtAMT3 may transport either at least two distinct compounds required for FeS and MoCo assembly in the cytosol, or a single compound required for the production of both cofactors. The central role of ATM3 in the production of MoCo was confirmed in a subsequent study, which showed that nitrate reductase and sulfite reductase activity, both of which require MoCo, were reduced to approximately 50 % from wild-type plants, while those which depend also on FeS were virtually undetectable (Fig. 1, Teschner et al. 2010). *Atatm3* mutants accumulate the first intermediate of MoCo synthesis, pyranopterin, which is produced in mitochondria. Nevertheless, the authors did not provide transport data and therefore the substrate(s) of ATM3 are still to be identified.

AtATM3 and a homologue from *Chlamydomonas*, CrCds1, have been shown to also play a role in cadmium tolerance (Hanikenne et al. 2005; Kim et al. 2006). In the case of *Chlamydomonas* the authors searched for mutants sensitive to cadmium. One of these mutants encoded for a HMT1 homologue. The mutant accumulated even more phytochelatins than the corresponding wild type and was also hypersensitive to iron. In order to identify *Arabidopsis* ABC transporters involved in heavy metal transport and/or resistance, Bovet et al. (2005) used a microarray specific for Arabidopsis ABC transporters. Among other genes, they observed that AtATM3/AtABCB25, was strongly upregulated in the roots of cadmium-treated plants. Overexpression of AtABCB25 enhanced Cd resistance, while a T-DNA insertion in this gene led to increased sensitivity (Kim et al. 2006). However, since *atatm3* mutants are already affected in their growth, the increased sensitivity might also reflect the decreased fitness of the *atatm3* mutant. Interestingly, when compared to

wild-type *atabcb25* mutants produce more glutathione in the presence of cadmium. This observation indicates that this mutant suffers higher oxidative stress and may be the link between the Fe–S, MoCo production and the cadmium phenotype.

3 Vacuolar ABCCs Are Involved in Heavy Metal Detoxification

The first ABC transporter of the ABCC family involved in heavy metal resistance was identified in *S. cerevisiae,* a fungus not producing phytochelatins. In the absence of Yeast Cadmium Factor 1 (YCF1), yeast is sensitive to cadmium (Szczypka et al. 1994) as well as to arsenic (As) and antimony (Sb) (Ghosh et al. 1999). It could be shown that YCF1 requires glutathione to confer heavy metal resistance and that YCF1-mediated detoxification of cellular heavy metals/metalloids occurs by transporting the glutathione-heavy metal complexes GS_2–Cd and GS_3–As, respectively (Li et al. 1997; Ghosh et al. 1999). A similar mode of action has been postulated for the human MRP1/HsABCC1, which partially complements the yeast mutant *ycf1* (Tommasini et al. 1996). It has also been suggested that detoxification of toxic heavy metals in plants may be partially mediated by glutathione heavy metal complexes mechanism, however, so far the importance of such a mechanism in plants is still elusive.

Plants are known to produce glutathione-derived heavy metal chelators, so-called phytochelatins that are produced in response to increased levels of cadmium, lead, arsenic, and some other heavy metals (Grill et al. 1989; Clemens 2006). Heavy metal–phytochelatin complexes are more stable compared to those including glutathione, and therefore they are more efficient in detoxifying potentially toxic heavy metals. Using vacuoles isolated from oat roots, Salt and Rauser (1995) provided evidence that apo-phytochelatin as well as Cd–phytochelatin is taken up by vacuoles in a strictly MgATP-dependent manner. While uptake of cadmium could be inhibited by both vanadate and NH_4^+, the latter of which abolishes pH gradients, phytochelatin transport was inhibited by vanadate only. These results indicated that cadmium uptake into the vacuole can be mediated by ABC transporters and H^+ gradient-dependent transporters, while phytochelatins are likely to be transported exclusively by an ABC transporter. Most ABC transporters found in the tonoplast belong to the ABCC clade. Expression studies showed that among the ABCC clade of *Arabidopsis*, it is mainly ABCC3, which is highly induced by cadmium (Bovet et al. 2005). Cadmium-dependent transcript increase was also observed for AtABCC6, 8, 10, and 12 (Bovet et al. 2005; Gaillard et al. 2008). Studies using T-DNA insertion lines for AtMRP3/AtABCC3 as well as AtAMRP6/AtABCC6 presented evidence that, indeed, these two ABC transporters are likely to be involved in cadmium resistance. AtABCC3 was able to partially complement the cadmium-sensitive phenotype of *ycf1* yeast (Tommasini et al. 1998), indicating that it could transport glutathione–cadmium complexes.

However, only a very slightly higher sensitivity was observed for the *atabcc3* mutant in the presence of cadmium (Klein and Martinoia, unpublished). While *AtABCC6* could not be expressed in yeast (Gaillard et al. 2008), the authors presented evidence that, at an early developmental stage, leaves of *atabcc6* knock-out mutants exhibited impaired growth in the presence of cadmium. No effect could be observed at the root (Gaillard et al. 2008). Nevertheless, the mechanism through which these two ABC transporters might be involved in heavy metal resistance remains elusive. Wang and Wu (2006) presented further evidence that in plants ABCCs are involved in heavy metal tolerance. These authors created insertional mutants in *Chlamydomonas reinhardtii* and screened them for cadmium sensitivity. Amongst them, the authors identified a mutant that carried a deletion of an ABCC-type transporter gene. This ABC transporter, *CrMRP2*, was strongly upregulated by cadmium and conferred cadmium tolerance when heterologously expressed in *ycf1* yeast, indicating that CrMRP2 acts as a glutathione–Cd transporter, although it cannot be excluded that phytochelatins may also be transported. Since this *Chlamydomonas* mutant is still producing similar amounts of phytochelatins as the wild type, CrMRP2 it is more likely to be a glutathione-transporter.

Based on the assumption that the vacuolar phytochelatin transporter should be a member of the ABCC family, Song et al. (2010) performed a large screen of T-DNA insertion mutants in all *Arabidopsis* ABCCs using arsenic as toxic heavy metalloid known to be detoxified by phytochelatins. The rationale behind this assumption was that only ABCCs had so far been demonstrated to unequivocally reside on the vacuolar membrane. Furthermore, two ABCC members had already been suggested to be involved in heavy metal detoxification (Tommasini et al. 1998; Gaillard et al. 2008). In the presence of arsenic-containing herbicide DSMA, *AtABCC1* and *AtABCC2* T-DNA insertion lines showed a slightly impaired root growth. However, the corresponding double mutant exhibited a very drastic phenotype and was extremely sensitive to arsenate and disodium methanearsonate (DSMA) when grown on both, agar and soil (Figs. 1 and 2b). A first indication that these transporters could indeed mediate the transfer of phytochelatins into the vacuole was gained from the observation that the arsenic sensitivity of *ycf1* yeast could be restored only by coexpressing a phytochelatine synthase and either AtABCC1 or AtABCC2. Transport experiments with vesicles isolated from yeast expressing either AtABCC1 or AtABCC2 revealed that both proteins transported apoPC and with an even higher capacity As-PC_2. The concentration-dependent uptake of As-PC_2 did not exhibit classical saturation kinetics but followed a sigmoid curve, indicating that As-PC_2 transport is regulated allosterically. This characteristic may be important to maintain a low cytosolic PCs pool under non-stress conditions, allowing for apoPCs to accumulate in the cytosol where they interact with heavy metals before being transported into the vacuole. Transport experiments performed with vacuoles isolated from *atabcc1 atabcc2* double knock-out *Arabidopsis* plants showed that these vacuoles exhibit a residual As(III)-PC_2 transport activity of only 10–15 %, indicating that these two ABC transporters are the major PC transporters in *Arabidopsis*. Overexpression of the transporters alone did not result in plants with an increased As tolerance, but the additional

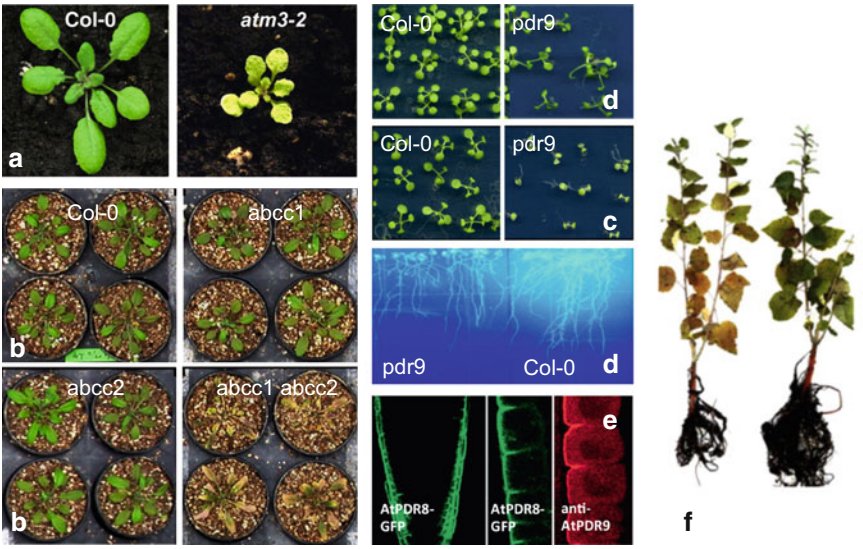

Fig. 2 Phenotypes and localization of ABC transporters involved in heavy metal allocation or detoxification. The *Arabidopsis* mutant of the mitochondrial ATM3 protein is chlorotic and exhibits strong growth defects (Teschner et al. 2010) (**a**). Phenotypes of wild-type *Arabidopsis* plants, the *atabcc1*, *atabcc2*, and *atabcc1xatabcc2* double mutants grown on arsenic-containing soil. Only the double mutant shows a high sensitivity against arsenic (Song et al. 2010) (**b**). *atabcg37*/ *pdr9* mutant plants are strongly impaired in growth and development when iron is a limiting factor in the medium (**c**, *lower panel*), while their growth is only slightly retarded under sufficient iron conditions (**c**, *upper panel*) (Fourcroy et al. 2013). This is due to a reduced iron mobilization as a consequence of impaired excretion of coumarylic compounds (**d**). AtABCG36/ PDR8 and AtABCG37/ PDR9 are localized on the lateral side of the rhizodermis, allowing for an efficient excretion of compounds to the soil (**e**) (Modified from Bätz and Martinoia 2014). Transgenic poplar trees transformed with the yeast YCF1 ABC transporter are less chlorotic than the corresponding wild-type trees and form a much larger and denser root system (Shim et al. 2013)

coexpression of phytochelatin synthase was necessary to attain this desired As-tolerant phenotype.

Subsequent experiments revealed that AtABCC1 and AtABCC2 also play a predominant role in conferring Cd and Hg(II) tolerance (Park et al. 2012). The *atabcc1 atabcc2* double knock-out displayed similar degrees of sensitivity to Hg (II) and arsenic. The sensitivity response in the presence of cadmium was strong but less pronounced compared to As and Hg, which was probably due to the fact that at pH 7 phytochelatin–cadmium complexes are very stable, while those with As(III) or Hg(II) are less stable. In summary, phytochelatin–cadmium complexes are stable in the cytosol, conferring a significant, although not complete level of tolerance to Cd without the ABCC transporters, whereas the phytochelatin–Hg and –As complexes absolutely require the ABCC transporters to be stably detoxified. The role of ABCC1 and ABCC2 in Cd sequestration is supported by the observation that in

cadmium-treated plants a Cd-sensing fluorescent signal marked the vacuole of wild-type plants, but marked the cytosol in *atabcc1 atabcc2* cells. The *atabcc1* single knock-out mutant was more sensitive than the wild type to Cd(II) and Hg(II), however by contrast, the *atabcc2* knockout mutant did not exhibit any dramatic difference in its sensitivity from the wild type. These results suggest that both AtABCC1 and AtABCC2 contribute to Cd(II) and Hg(II) tolerance, and that AtABCC1 can confer a significant level of tolerance to these divalent heavy metals in the absence of AtABCC2. Most of the work on ABCCs, phytochelatins, and heavy metals has been done with *Arabidopsis*. A recent work performed with barley vacuoles (Song et al. 2014) indicates that although the general mechanism for PCs-As transport is conserved in *Arabidopsis* and barley, the transport characteristics for PCs–heavy metal complexes may be different between plant species, and that PC-essential heavy metal complexes can be formed and transported into vacuole by ABC transporters. Together with the previous report suggesting a role of phytochelatin in Zn homeostasis (Tennstedt et al. 2009), this raises the question how plant cells can maintain essential heavy metal homeostasis when they take up non-essential, toxic heavy metals.

Interestingly, it has been shown in rice that a P-type ATPase, HMA3, is the major vacuolar transporter conferring cadmium resistance (Ueno et al. 2010). Its *Arabidopsis* homologue, AtHMA3, acts mainly as a zinc transporter, and the cadmium phenotype observed for the corresponding mutant plants is relatively weak (Morel et al. 2009). However, OsHMA3 has a far higher specificity for cadmium than AtHMA3 and the corresponding mutant is highly sensitive to cadmium. It will therefore be of interest to investigate the role of phytochelatin-transporters in rice and to investigate their role in cadmium tolerance. A tonoplast-localized ABC transporter in rice is involved in aluminum tolerance (Huang et al. 2012). OsALS1, a close homologue to the animal TAP peptide transporters is a member of ABCB subfamily and expressed mainly in roots (Fig. 1). A loss-of-function mutant of *osals1* exhibited sensitivity specifically to Al but not to other heavy metals. Although no Al transport activity of OsALS1 could be detected visual examination of Al using morin staining suggested that Al levels increase in the cytoplasm of *osals1* mutant roots. An *Arabidopsis* homolog, AtALS1, is also localized to the tonoplast, but the mutant phenotype of AtALS1 is limited to narrow ranges of Al concentrations (Larsen et al. 2007).

4 Plasma Membrane-Localized ABC Transporters Involved in Heavy Metals Resistance and Uptake

Transport processes mediating detoxification of Na^+ occur at both the vacuolar membrane as well as at the plasma membrane. Internal and external excretion processes are responsible for keeping Na^+ at nontoxic concentrations. Na^+/H^+ antiporters are responsible for the accumulation of Na^+ within the vacuole, where

it acts as "cheap" osmoticum. Excretion back into the apoplast and finally into the soil ensures the maintenance of nontoxic, intracellular Na^+ levels (Zhu 2002). Although, as described above, ABC transporters are involved in vacuolar deposition of heavy metals, to date no direct export of heavy metals from root to soil has been documented to our knowledge. Nevertheless, two plasma membrane-localized ABCGs and one bacterial-type ABC protein play an important role in heavy metal tolerance.

The best characterized among them are the bacterial-type ABC proteins OsSTAR1 and OsSTAR2 that play an important role in Aluminum (Al) tolerance in rice. Al is a major toxic metal that limits crop production in acidic soil (Fig. 1). A well-known mechanism to cope with aluminum toxicity is the excretion of citrate by MATE transporters (Magalhaes et al. 2007) and malate secretion by ALMTs into the rhizosphere (Delhaize et al. 2004; Meyer et al. 2010). These organic acids chelate Al, preventing the entry of the toxic Al^{3+} into the root (Ryan et al. 2011). OsSTAR1 and OsSTAR2 were identified in a screen to discover new genes involved in Al tolerance (Huang et al. 2009). As is often observed in bacteria, the cytosolic and membrane domains of an ABC transporter are encoded by two different genes. OsSTAR1 corresponds to the nucleotide-binding domain of the transporter and OsSTAR2 to its membrane domain. The combined ABC transporter complex was shown to locate mainly to membrane vesicles of root cells. Coexpression of OsSTAR1 and OsSTAR2 in oocytes of *Xenopus laevis* revealed that both proteins form a functional ABC transporter able to transport UDP-glucose. The discovery that UDP-glucose is involved in aluminum tolerance was surprising. The authors suggested that UDP-glucose may be used to alter the composition of the cell wall, thus avoiding migration of Al into proximity of the plasma membrane. The same group reported that a close homologue, AtSTAR1 exists in *Arabidopsis* (Huang et al. 2010). The knockout mutant of AtSTAR1 (ABCI17) was also sensitive to aluminum. The observation that OsSTAR1 could rescue the aluminum sensitive phenotype of the *Arabidopsis* mutant indicates that both genes code for proteins exhibiting a similar function.

In the transcriptome-based screen described above, Bovet et al. (2005) discovered that AtPDR8/AtABCG36/PEN3 is highly upregulated by cadmium. Further studies showed that this is also true for lead. Like all full-size ABCG transporters described so far, AtPDR8/AtABCG36 is localized at the plasma membrane (Kobae et al. 2006; Stein et al. 2006). *Arabidopsis* plants overexpressing PDR8 were more resistant to cadmium and lead, while RNAi and T-DNA mutant plants were more sensitive to it (Fig. 1, Kim et al. 2007). At the same time, plants overexpressing AtPDR8 contained less cadmium, while RNAi/T-DNA mutants contained more. Flux experiments using Arabidopsis mesophyll protoplasts indicated that Cd^{2+} export was more pronounced in AtPDR8 overexpressing plants than in the corresponding mutants. This result indicates that AtPDR8 is able to export Cd, however, whether this export involves Cd as Cd^{2+} or a Cd complex is yet unknown. Even an indirect activation of a Cd efflux transporter cannot be excluded. Interestingly, AtPDR8/AtABCG36/PEN3 transporter has been shown to play an important role in plant pathogen defense (Kobae et al. 2006; Stein et al. 2006). It will be

therefore interesting to see, whether the substrate(s) conferring heavy metal resistance and the ones involved in the plant–pathogen reaction are the same, or whether AtABCG36, as other ABC transporters, transports structurally unrelated compounds (Kang et al. 2011) and exerts its dual function in this way.

In a screen aimed to identify whether ABC transporters could be involved in lead detoxification, Lee et al. (2005) (Fig. 1) observed that the transcript levels of AtPDR12/AtABCG40 increased when Pb was present in the medium. As for AtPDR8, plants overexpressing AtPDR12 were more resistant to Pb^{2+} and accumulated less of the heavy metal, while knockout mutants of this transporter were more sensitive and accumulated more Pb^{2+}. The observation that inhibition of glutathione biosynthesis had a much stronger effect than the absence of AtABCG40 indicates that there is an additional glutathione-dependent detoxification pathway that plays a more important role than that mediated by AtABCG40. Later it was found that ABCG40 specifically transports abscisic acid (ABA) (Kang et al. 2010). Since Pb did not compete with ABA for transport via ABCG40 (Kang and Lee, unpublished result), the role of the transporter in Pb tolerance might be indirect via uptake of the stress hormone ABA. However, it cannot be excluded that ABCG40 might also transport Pb directly, since some ABC transporters can transport diverse substrates of different structures (Kang et al. 2011).

Iron deficiency is often paralleled by the upregulation of genes of the phenylpropanoid pathway and release of phenolics from the root to the soil (Schmid et al. 2014). Recently it has been shown that *Arabidopsis* plants that cannot excrete phenolics are more sensitive to iron starvation under conditions of low iron availability. Coexpression studies showed that AtABCG37 was strongly upregulated by iron deficiency (Fourcroy et al. 2013; Rodríguez-Celma et al. 2013) and it was therefore questioned whether this transporter exports phenolics that may complex and solubilize Fe. Indeed AtABCG37 mutants were impaired in their growth under iron limiting conditions (Figs. 1 and 2c). In a careful study Fourcroy et al. (2013) could show that AtABCG37 was required for the secretion of coumarin compounds and hence to supply iron to plants under conditions of sparse iron availability (Fig. 2d). Their role in root excretion is underlined by the observation that AtABCG37, as ABCG36 exhibits a polar localization on the distal part of the rhizodermis (Fig. 2e). However, ABCG37 was also reported to transport auxinic compounds, including the auxin precursor, IBA (Růžička et al. 2010; see chapter "IBA Transport by PDR Proteins").

5 ABC Transporters and Their Potential in Heavy Metal Phytoremediation

Heavy metal-contaminated soils pose a problem for food security in many parts of the world. On the one side, there is a natural presence or deposition of heavy metals in certain regions. On the other side, industrialization and mining have

contaminated large regions worldwide. In reality, the main contaminants are cadmium and arsenic. For over 20 years, scientists have tried to develop plants that can efficiently purify soils (Clemens et al. 2002; Verbruggen et al. 2009; Singh et al. 2011). Plants that can accumulate large amounts of heavy metals, so-called hyper-accumulators, have been known for a long time. However, these plants produce only very small biomass and are therefore not suited to purify soils (Krämer 2010). Hence, the goal of scientists is to either find plants that produce high biomass and that at the same time accumulate considerable amounts of heavy metals, or produce transgenic plants that display these features. Many approaches have been taken to either produce higher amounts of glutathione or phytochelatins in plants, or to express transporters that allocate more heavy metals either to the shoot or to the vacuole.

Since some ABC transporters have been shown to play an important role in heavy metal resistance: and accumulation, several approaches were employed to take advantage of their properties: Song et al. (2003) introduced the *Saccaromyces YCF1* into *Arabidopsis*. The authors could show that, as in yeast, YCF1 is targeted to the vacuolar membrane in *Arabidopsis*. Plants expressing YCF1 were more tolerant to cadmium and lead and accumulated significantly more of these heavy metals. Transport studies with isolated vacuoles revealed that, indeed, uptake of GS_2-Cd was strongly increased in the transgenic lines, demonstrating that this transport activity could be exploited to create more heavy metal accumulating plants.

In a subsequent work, Bhuiyan et al. (2011a) introduced *YCF1* in the partially cadmium-resistant crop plant *Brassica juncea*. As in *Arabidopsis* they could observe that the transgenic plants were slightly more tolerant to cadmium and lead and also accumulated up to twofold more cadmium compared to the control plants. *YCF1* has also been introduced into poplar, and transgenic poplar plants have been tested on soil contaminated with several heavy metals under greenhouse and field conditions (Shim et al. 2013). Poplar plants expressing YCF1 were significantly more tolerant to the contaminated soil compared to control plants, produced less necrotic spots, and accumulated up to three times more cadmium under greenhouse conditions. In the field, transgenic plants were still less chlorotic and produced a higher biomass, although the increase in cadmium content was less pronounced compared to greenhouse conditions (Fig. 2f). The YCF1-expressing poplar trees are unlikely to be useful for removing heavy metals from the contaminated soil rapidly and efficiently, due to the low bio-concentration values. Nevertheless, these plants may prove valuable in long-term stabilization of polluted soils such as can be found around mining sites. Transgenic poplar trees could establish larger and more ramified root systems, which can be expected to bind and fortify the soil, thus slowing down erosion and spreading of pollutants. Moreover, the transgenic trees tolerated and accumulated multiple heavy metals and metalloids, such as As, Zn, and Pb and can therefore better survive heterogeneous contaminations as can be found on closed-mine sites than the corresponding control plants. Besides phytostabilization, the trees might actually be able to perform phytoextraction of the heavy metals from the polluted sites if allowed to grow during their whole life

span of more than 30 years. Taking trees has also the advantage that they do not require much maintenance.

As mentioned above, parallel overexpression of AtABCC1/AtABCC2 and PCS results in plants with an increased resistance to arsenic (Song et al. 2010). However, also here the effect is marginal. As already discussed above, overexpression of AtATM3 in *Arabidopsis* led to plants exhibiting a higher cadmium tolerance (Kim et al. 2007). Similar results were obtained when AtATM3 was overexpressed in *Brassica juncea* (Bhuiyan et al. 2011b). Growth of the transgenic plants was less impaired in the presence of cadmium and lead, and a transgenic plant accumulated approximately two times more cadmium. This result is surprising, since it contrasts results from ATM3-overexpressing Arabidopsis plants; they contained less cadmium, while T-DNA mutants or RNAi lines contained more when compared to control plants. However, AtATM3-overexpressing *Brassica* displayed an upregulation of genes involved in glutathione synthesis as well as of genes of some heavy metal transporters. Therefore, the authors hypothesized that the higher cadmium contents in AMT3-overexpressing *Brassica* were due to a modulation of the glutathione-synthesis and heavy metal transporter genes. In fact, to date, we have no knowledge about the exact transport mechanism and substrate specificity of AtATM3 and the use of this ABC transporter in phytoremediation approaches may probably be premature.

In conclusion, overexpression of YCF1 and AtATM3 increases cadmium and lead tolerance and accumulation. AtABCC1/2 has been shown to increase Cd tolerance and accumulation (Song et al. 2010; Park et al. 2012). However, the degree of accumulation is far too small for these plants to be suitable for fast and efficient phytoextraction. For efficient phytoremediation, different strategies have to be combined, where mobilization of heavy metals from the soil, uptake, allocation to the areal part and finally chelating and deposition into the vacuole can be efficiently achieved.

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References

Bätz U, Martinoia E (2014) Root exudates: the hidden part of plant defense. Trends Plant Sci 19:90–98

Bernard DG, Cheng Y, Zhao Y, Balk J (2009) An allelic mutant series of ATM3 reveals its key role in the biogenesis of cytosolic iron-sulfur proteins in Arabidopsis. Plant Physiol 151:590–602

Bhuiyan MSU, Min SR, Jeong WJ, Sultana S, Choi KS, Lee Y, Liu JR (2011a) Overexpression of AtATM3 in Brassica juncea confers enhanced heavy metal tolerance and accumulation. Plant Cell Tiss Org Cult 107(1):69–77

Bhuiyan MSU, Min SR, Jeong WJ, Sultana S, Choi KS, Song WY, Lee Y, Lim YP, Liu JR (2011b) Overexpression of a yeast cadmium factor 1 (YCF1) enhances heavy metal tolerance and accumulation in Brassica juncea. Plant Cell Tiss Org Cult 105:85–91

Bovet L, Feller U, Martinoia E (2005) Possible involvement of plant ABC transporters in cadmium detoxification: a cDNA sub-microarray approach. Environ Int 31:263–267

Burkhead JL, Reynolds KA, Abdel-Ghany SE, Cohu CM, Pilon M (2009) Copper homeostasis. New Phytol 182:799–816

Chen S, Sánchez-Fernández R, Lyver ER, Dancis A, Rea PA (2007) Functional characterization of AtATM1, AtATM2, and AtATM3, a subfamily of Arabidopsis half-molecule ATP-binding cassette transporters implicated in iron homeostasis. J Biol Chem 282:21561–21571

Clemens S (2006) Evolution and function of phytochelatin synthases. J Plant Physiol 163:319–332

Clemens S, Palmgren MG, Kramer UA (2002) Long way ahead: understanding and engineering plant metal accumulation. Trends Plant Sci 7:309–315

Cobbett CS (2000) Phytochelatin biosynthesis and function in heavy-metal detoxification. Curr Opin Plant Biol 3:211–216

Delhaize E, Ryan PR, Hebb DM, Yamamoto Y, Sasaki T, Matsumoto H (2004) Engineering high-level aluminum tolerance in barley with the ALMT1 gene. Proc Natl Acad Sci USA 101:15249–15254

Fourcroy P, Sisó-Terraza P, Sudre D, Savirón M, Reyt G, Gaymard F, Abadía A, Abadia J, Alvarez-Fernández A, Briat JF (2013) Involvement of the ABCG37 transporter in secretion of scopoletin and derivatives by Arabidopsis roots in response to iron deficiency. New Phytol 201:155–167

Gaillard S, Jacquet H, Vavasseur A, Leonhardt N, Forestier C (2008) AtMRP6/AtABCC6, an ATP-binding cassette transporter gene expressed during early steps of seedling development and up-regulated by cadmium in *Arabidopsis thaliana*. BMC Plant Biol 8:22–32

Ghosh M, Shen J, Rosen BP (1999) Pathways of As(III) detoxification in Saccharomyces cerevisae. Proc Natl Acad Sci USA 96:5001–5006

Grill E, Loffler S, Winnacker EL, Zenk MH (1989) Phytochelatins, the heavy-metal-binding peptides of plants, are synthesized from glutathione by a specific gamma-glutamylcysteine dipeptidyl transpeptidase (phytochelatin synthase). Proc Natl Acad Sci U S A 86:6838–6842

Hanikenne M, Motte P, Wu MCS, Wang T, Loppes R, Matagne RF (2005) A mitochondrial half-size ABC transporter is involved in cadmium tolerance in Chlamydomonas reinhardtii. Plant Cell Environ 28:863–873

Huang CF, Yamaji N, Mitani N, Yano M, Nagamura Y, Ma JF (2009) A bacterial-type ABC transporter is involved in aluminum tolerance in rice. Plant Cell 21:655–667

Huang CF, Yamaji N, Ma JF (2010) Knockout of a bacterial-type ABC transporter gene, *AtSTAR1*, results in increased Al sensitivity in Arabidopsis. Plant Physiol 153:1669–1677

Huang CF, Yamaji N, Chen Z, Ma JF (2012) A tonoplast-localized half-size ABC transporter is required for internal detoxification of aluminum in rice. Plant J 69:857–867

Hussain D, Haydon MJ, Wang Y, Wong E, Sherson SM, Young J, Camakaris J, Harper JF, Cobbett CS (2004) P-type ATPase heavy metal transporters with roles in essential zinc homeostasis in Arabidopsis. Plant Cell 16:1327–1339

Kang J, Hwang JU, Lee M, Kim YY, Assmann SM, Martinoia E, Lee Y (2010) PDR-type ABC transporter mediates cellular uptake of the phytohormone abscisic acid. Proc Natl Acad Sci U S A 107:2355–2360

Kang J, Park J, Choi H, Burla B, Kretzschmar T, Lee Y, Martinoia E (2011) Plant ABC transporters. The Arabidopsis book. BioOne 9:e0153

Kim DY, Bovet L, Kushnir S, Noh EW, Martinoia E, Lee Y (2006) AtATM3 is involved in heavy metal resistance in Arabidopsis. Plant Physiol 140:922–932

Kim DY, Bovet L, Maeshima M, Martinoia E, Lee Y (2007) The ABC transporter AtPDR8 is a cadmium extrusion pump conferring heavy metal resistance. Plant J 50:207–218

Kobae Y, Sekino T, Yoshioka H, Nakagawa T, Martinoia E, Maeshima M (2006) Loss of AtPDR8, a plasma membrane ABC transporter of *Arabidopsis thaliana*, causes hypersensitive cell death upon pathogen infection. Plant Cell Physiol 47:309–318

Krämer U (2010) Metal hyperaccumulation in plants. Annu Rev Plant Biol 61:517–534

Kushnir S, Babiychuk E, Storozhenko S, Davey MW, Papenbrock J, De Rycke R, Engler G, Stephan UW, Lange H, Kispal G (2001) A mutation of the mitochondrial ABC transporter Sta1 leads to dwarfism and chlorosis in the Arabidopsis mutant starik. Plant Cell 13:89–100

Larsen PB, Cancel J, Rounds M, Ochoa V (2007) Arabidopsis ALS1 encodes a root tip and stele localized half type ABC transporter required for root growth in an aluminum toxic environment. Planta 225:1447–1458

Lee M, Lee K, Lee J, Noh EW, Lee Y (2005) AtPDR12 contributes to lead resistance in Arabidopsis. Plant Physiol 138:827–836

Leighton J, Schatz G (1995) An ABC transporter in the mitochondrial inner membrane is required for normal growth of yeast. EMBO J 14:188–195

Li ZS, Lu YP, Zhen RG, Szczypka M, Thiele DJ, Rea PA (1997) A new pathway for vacuolar cadmium sequestration in *Saccharomyces cerevisiae*: YCF1-catalyzed transport of bis (glutathionato)cadmium. Proc Natl Acad Sci U S A 94:42–47

Magalhaes JV, Liu J, Guimaraes CT, Lana UGP, Alves VMC, Wang YH, Schaffert RE, Hoekenga OA, Pineros MA, Shaff JE (2007) A gene in the multidrug and toxic compound extrusion (MATE) family confers aluminum tolerance in sorghum. Nat Genet 39:1156–1161

Martinoia E, Maeshima M, Neuhaus HE (2007) Vacuolar transporters and their essential role in plant metabolism. J Exp Bot 58:83–102

Martinoia E, Meyer S, DeAngeli A, Nagy R (2012) Vacuolar transporters in their physiological context. Annu Rev Plant Biol 63:183–214

Meharg AA, Rahman MM (2003) Arsenic contamination of Bangladesh paddy field soils: implications for rice contribution to arsenic consumption. Environ Sci Technol 37:229–234

Meyer S, De Angeli A, Fernie AR, Martinoia E (2010) Intra-and extra-cellular excretion of carboxylates. Trends Plant Sci 15:40–47

Morel M, Crouzet J, Gravot A, Auroy P, Leonhardt N, Vavasseur A, Richaud P (2009) AtHMA3, a P1B-ATPase allowing Cd/Zn/Co/Pb vacuolar storage in Arabidopsis. Plant Physiol 149: 894–904

Morrissey J, Baxter IR, Lee J, Li L, Lahner B, Grotz N, Kaplan J, Salt D, Guerinot ML (2009) The Ferroportin metal efflux proteins function in iron and cobalt homeostasis in Arabidopsis. Plant Cell 21:3326–3338

Napolitano M, Rubio MA, Santamaria-Gomez J, Olmedo-Verd E, Robinson NJ, Luque I (2012) Characterization of the response to zinc deficiency in the Cyanobacterium Anabaena sp strain PCC 7120. J Bacteriol 194:2426–2436

Ortiz DF, Kreppel L, Speiser DM, Scheel G, McDonald G, Ow DW (1992) Heavy metal tolerance in the fission yeast requires an ATP-binding cassette-type vacuolar membrane transporter. EMBO J 11:3491–3499

Ortiz DF, Ruscitti T, McCue KF, Ow DW (1995) Transport of metal-binding peptides by HMT1, a fission yeast ABC-type vacuolar membrane protein. J Biol Chem 270:4721–4728

Palmer CM, Guerinot ML (2009) Facing the challenges of Cu, Fe and Zn homeostasis in plants. Nat Chem Biol 5:333–340

Park J, Song WY, Ko D, Eom Y, Hansen TH, Schiller M, Lee TG, Martinoia E, Lee Y (2012) The phytochelatin transporters AtABCC1 and AtABCC2 mediate tolerance to cadmium and mercury. Plant J 69:278–288

Preveral S, Gayet L, Moldes C, Hoffmann J, Mounicou S, Gruet A, Reynaud F, Lobinski R, Verbavatz JM, Vavasseur A, Forestier C (2009) A common highly conserved cadmium detoxification mechanism from bacteria to humans: heavy metal tolerance conferred by the ATP-binding cassette (ABC) transporter SpHMT1 requires glutathione but not metal-chelating phytochelatin peptides. J Biol Chem 284:4936–4943

Rea PA (2007) Plant ATP-binding cassette transporters. Annu Rev Plant Biol 58:347–375

Rodríguez-Celma J, Lin WD, Fu GM, Abadía J, López-Millán AF, Schmidt W (2013) Mutually exclusive alterations in secondary metabolism are critical for the uptake of insoluble iron compounds by Arabidopsis and Medicago truncatula. Plant Physiol 162:1473–1485

Růžička K, Strader LC, Bailly A, Yang H, Blakeslee J, Łangowski L, Nejedlá E, Fujita H, Ito H, Syōno K, Hejátko J, Gray WM, Martinoia E, Geisler M, Bartel B, Murphy A, Friml J (2010) Arabidopsis PIS1 encodes the ABCG37 transporter of auxinic compounds including the auxin precursor indole-3-butyric acid. Proc Natl Acad Sci USA 107:10749–10753

Ryan P, Tyerman S, Sasaki T, Furuichi T, Yamamoto Y, Zhang W, Delhaize E (2011) The identification of aluminium-resistance genes provides opportunities for enhancing crop production on acid soils. J Exp Bot 62:9–20

Salt DE, Rauser WE (1995) MgATP-dependent transport of phytochelatins across the tonoplast of oat roots. Plant Physiol 107:1293–1301

Schmid NB, Giehl RN, Döll S, Mock H-P, Strehmel N, Scheel D, Kong X, Hider RC, von Wirén N (2014) Feruloyl-CoA 6′-Hydroxylase1-dependent coumarins mediate iron acquisition from alkaline substrates in Arabidopsis. Plant Physiol 164:160–172

Schwartz MS, Benci JL, Selote DS, Sharma AK, Chen AGY, Dang H, Fares H, Vatamaniuk OK (2010) Detoxification of multiple heavy metals by a half-molecule ABC transporter, HMT-1, and coelomocytes of Caenorhabditis elegans. PLoS One 5:e9564

Self WT, Grunden AM, Hasona A, Shanmugam KT (2003) Molybdate transport. Res Microbiol 152:311–321

Shikanai T, Müller-Moulé P, Munekage Y, Niyogi KK, Pilon M (2003) PAA1, a P-type ATPase of Arabidopsis, functions in copper transport in chloroplasts. Plant Cell 15:1333–1346

Shim D, Kim S, Choi Y-I, Song W-Y, Park J, Youk ES, Jeong S-C, Martinoia E, Noh E-W, Lee Y (2013) Transgenic poplar trees expressing yeast cadmium factor 1 exhibit the characteristics necessary for the phytoremediation of mine tailing soil. Chemosphere 90:1478–1486

Singh BR, Gupta SK, Azaizeh H, Shilev S, Sudre D, Song W-Y, Martinoia E, Mench M (2011) Safety of food crops on land contaminated with trace elements. J Sci Food Agric 91:1349–1366

Song WY, Sohn EJ, Martinoia E, Lee YJ, Yang YY, Jasinski M, Forestier C, Hwang I, Lee Y (2003) Engineering tolerance and accumulation of lead and cadmium in transgenic plants. Nat Biotechnol 21:914–919

Song WY, Park J, Mendoza-Cozatl D, Suter-Grotemeyer M, Shim D, Hortensteiner S, Geisler M, Weder B, Rea P, Rentsch D, Schroder J, Lee Y, Martinoia E (2010) Arsenic tolerance in Arabidopsis is mediated by two ABCC-type phytochelatin transporters. Proc Natl Acad Sci USA 107:21187–21192

Song WY, Mendoza-Cózatl DG, Lee Y, Schroeder JI, Ahn S-N, Lee H-S, Wicker T, Martinoia E (2014) Phytochelatin-metal(loid) transport into vacuoles shows different substrate preferences in Barley and Arabidopsis. Plant Cell Environ 37(5):1192–1201

Sooksa-Nguan T, Yakubov B, Kozlovskyy VI, Barkume CM, Howe KJ, Thannhauser TW, Rutzke MA, Hart JJ, Kochian LV, Rea PA (2009) Drosophila ABC transporter, DmHMT-1, confers tolerance to cadmium: DmHMT-1 and its yeast homolog, SpHMT-1, are not essential for vacuolar phytochelatin sequestration. J Biol Chem 284:354–362

Stein M, Dittgen J, Sánchez-Rodríguez C, Hou BH, Molina A, Schulze-Lefert P, Lipka V, Somerville S (2006) Arabidopsis PEN3/PDR8, an ATP binding cassette transporter, contributes to nonhost resistance to inappropriate pathogens that enter by direct penetration. Plant Cell 18:731–746

Szczypka MS, Wemmie JA, Moye-Rowley WS, Thiele DJ (1994) A yeast metal resistance protein similar to human cystic fibrosis transmembrane conductance regulator (CFTR) and multidrug resistance-associated protein. J Biol Chem 269:22853–22857

Tennstedt P, Peisker D, Böttcher C, Trampczynska A, Clemens S (2009) Phytochelatin synthesis is essential for the detoxification of excess zinc and contributes significantly to the accumulation of zinc. Plant Physiol 149:938–948

Teschner J, Lachmann N, Schulze J, Geisler M, Selbach K, Santamaria-Araujo J, Balk J, Mendel RR, Bittner F (2010) A novel role for Arabidopsis mitochondrial ABC transporter ATM3 in molybdenum cofactor biosynthesis. Plant Cell 22:468–480

Tommasini R, Evers R, Vogt E, Mornet C, Zaman GJ, Schinkel AH, Borst P, Martinoia E (1996) The human multidrug resistance-associated protein functionally complements the yeast cadmium resistance factor 1. Proc Natl Acad Sci USA 93:6743–6748

Tommasini R, Vogt E, Fromenteau M, Hortensteiner S, Matile P, Amrhein N, Martinoia E (1998) An ABC-transporter of *Arabidopsis thaliana* has both glutathione-conjugate and chlorophyll catabolite transport activity. Plant J 13:773–780

Ueno D, Yamaji N, Kono I, Huang CF, Ando T, Yano M, Ma JF (2010) Gene limiting cadmium accumulation in rice. Proc Natl Acad Sci USA 107:16500–16505

Vatamaniuk OK, Bucher EA, Sundaram MV, Rea PA (2005) CeHMT-1, a putative phytochelatin transporter, is required for cadmium tolerance in *Caenorhabditis elegans*. J Biol Chem 280:23684–23690

Verbruggen N, Hermans C, Schat H (2009) Mechanisms to cope with arsenic or cadmium excess in plants. Curr Opin Plant Biol 12:364–372

Verret F, Gravot A, Auroy P, Leonhardt N, David P, Nussaume L, Vavasseur A, Richaud P (2004) Overexpression of AtHMA4 enhances root-to-shoot translocation of zinc and cadmium and plant metal tolerance. FEBS Lett 576:306–312

Vert G, Grotz N, Dedaldechamp F, Gaymard F, Guerinot ML, Briat J, Curie C (2002) IRT1, an Arabidopsis transporter essential for iron uptake from the soil and for plant growth. Plant Cell 14:1223–1233

Wang TL, Wu M (2006) An ATP-binding cassette transporter related to yeast vacuolar ScYCF1 is important for Cd sequestration in Chlamydomonas reinhardtii. Plant Cell Environ 29:1901–1912

Williams PN et al (2005) Variation in arsenic speciation and concentration in paddy rice related to dietary exposure. Environ Sci Technol 39:5531–5540

Zhao FJ, McGrath SP, Meharg AA (2010) Arsenic as a food chain contaminant: mechanisms of plant uptake and metabolism and mitigation strategies. Annu Rev Plant Biol 61:535–559

Zhu J-K (2002) Salt and drought stress signal transduction in plants. Annu Rev Plant Biol 53: 247–273

Phytate Transport by MRPs

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Abstract Phytic acid is the main storage form for phosphate in plant seeds. From a nutritional point of view it decreases the seed value by chelating important minerals, such as iron, zinc, magnesium, and calcium. Therefore, the isolation of low phytic acid (*lpa*) mutants is considered a highly desirable objective in the genetic improvement of the nutritional quality of grain crops. On the other side phytic acid is a very important signaling molecule involved in development and hormonal regulation. The only phytic acid transporters characterized so far are proteins of the ABCC type. Some *lpa* mutants affected in these transporters have been isolated and characterized in Arabidopsis and in crops. Here we review advances in the characterization of these proteins and on the corresponding mutants. Moreover we propose an explanation on how mutations in these transporters may affect different aspects of cellular metabolism not only strictly related to phytic acid biosynthesis.

1 Phytic Acid

Phytic acid (*myo*-inositol-1,2,3,4,5,5-hexa*kis*phosphate; $InsP_6$) is a ubiquitous component of eukaryotic cells. In plants it is the most abundant form in which phosphorus is accumulated in seeds (up to 85 % of total phosphorus) and other plant tissues and organs such as pollen, roots, tubers, and turions. During germination, phytase enzymes remobilize the phosphorus stored as $InsP_6$ to support seedling growth (Raboy 2003). Due to its chemical structure (highly negatively charged at physiological pH), $InsP_6$ easily precipitates in the form of phytate salts binding important mineral cations such as iron, zinc, potassium, calcium, and magnesium.

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Since monogastric animals and humans lack phytases in their digestive tract, $InsP_6$ is poorly digested and decreases the nutritional value of the seeds by limiting phosphorus and mineral bioavailability. As a consequence, high amounts of undigested phytates are released with the animal waste into the environment, thus accentuating the phosphorus pollution from agriculture, whereas poor mineral bioavailability is ascribed as one of the most important causes of mineral deficiencies (mainly iron and zinc) in those populations whose diet is largely based on staple crops (Raboy 2001).

To improve the nutritional value of seeds, plant breeders have spent many efforts to isolate and develop *low phytic acid* (*lpa*) crops and a number of such mutants, in which a 45–90 % reduction of phytic acid was achieved, have been obtained (Raboy 2009). A consistent number of these *lpa* mutants bears mutations in a gene coding for an ABCC-type transporter, orthologous to the Arabidopsis *AtMRP5* gene which recently was demonstrated to code for a high-affinity $InsP_6$ ATP-binding cassette transporter (Shi et al. 2007; Xu et al. 2009; Nagy et al. 2009; Gillman et al. 2009; Panzeri et al. 2011). Interestingly a screen for *lpa* mutations of a maize EMS mutagenized seed population revealed that the *ZmMRP4* locus, coding for such a transporter, is highly mutable with a rate nearly an order of magnitude greater than a typical rate (Raboy 2009) and that 10 % of the mutations in this locus were lethal (Raboy et al. 2001). Similar phenomenon may explain in part the high rate of mutation observed for this locus in different crops.

2 Structure, Expression, and Subcellular Localization of MRP Type ABC Phytic Acid Transporters

Phytic acid transporters are multidrug resistance-associated proteins (MRPs), belonging to the ABCC cluster of plant ABC transporters (Verrier et al. 2008). They are full-length ABC transporters containing the classical two membrane-spanning domains (TMD1 and TMD2, each containing six transmembrane α-helices), which constitute the membrane- spanning pore, tandemly arranged with two cytosolic domains, referred as nucleotide-binding domains (NBD1 and NBD2). These last contain the Walker A and B motifs together with the characteristic ABC signature. The domain arrangement is in the so-called forward orientation: TMD1-NBD1_TMD2-NBD2 (Fig. 1). $InsP_6$ transporters, as other members of the ABCC cluster, contain an additional extremely hydrophobic N-terminal extension (TMD0, containing five transmembrane α-helices), which is connected to the rest of the protein via a cytosolic loop (CL3), rich in charged amino acids. The role of this N-terminal TMD0 in plants is unknown; however, studies on some human and yeast ABCC type transporters suggest it might be important for appropriate protein trafficking and targeting (Mason and Michaelis 2002; Westlake et al. 2005; Bandler et al. 2008). As the cytosolic loop CL3 is concerned, it has been shown to play an important role in substrate recognition and transport (Gao et al. 1998).

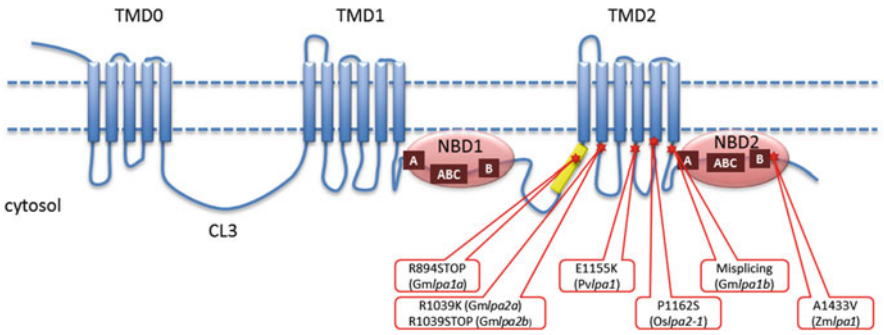

Fig. 1 Schematic representation of the structure of the MRP-type ABCC phytic acid transporter. The different domains are as follows: *red*, NBDs 1 and 2; *blue*, TMDs 0, 1 and 2; CL3, cytosolic loop domain; *dark red squares*, Walker A and B domains (A, B) and ABC signature (ABC); *yellow rectangle*, conserved stretch of lysine residues; *red stars*, position of the known mutations reported for *lpa*-MRP-type ABCC phytic acid mutants

The first evidence that an MRP-type ABC transporter was required for $InsP_6$ transport and accumulation in seed was provided by Shi and coworkers (Shi et al. 2007), using the maize insertional *lpa1* mutants in the *ZmMRP4* gene, while the biochemical demonstration was given by Nagy and coworkers using the Arabidopsis homolog AtMRP5 (also referred to as AtABCC5) (Nagy et al. 2009). They performed [^{33}P] $InsP_6$ transport uptake studies on microsomes obtained from a *ycf1* yeast mutant (a model system commonly used to investigate the function of ABC transporters) carrying the AtMRP5 transporter. Phytic acid transport was dependent on the presence of AtMRP5 and strictly dependent on the addition of ATP. The kinetics analysis of the transport showed V_{max} values of about 1.6–2.5 μmol min^{-1} mg^{-1} and a K_m ranging between 263 and 310 nm, indicating that AtMRP5 has a very high affinity for phytic acid. Further confirmation of the ATP-dependent activity of the transporter was provided showing that disruption by site directed mutagenesis of specific residues in the Walker B domain of NBD1 and NBD2 reproduced the *mrp5* phenotype (Nagy et al. 2009).

At present, it is not known which amino acid residues are specifically involved in phytic acid transport. In fact, the amino acids compromised in known *lpa* mutants (red stars in Fig. 1) are conserved also in other ABCC proteins, indicating they should be involved in general proper functioning of ABCC transporters. This is clearly the case of the mutation A1433V found in the maize *lpa1-1*, which is located in the putative D-loop sequence (ASVD), adjacent to the Walker B motif (ILVLD), of the NBD2 domain (ILVLDEATA$_{1433}$SVD).

A detailed analysis of the multiple alignment of the amino acid sequence of MRP-type $InsP_6$ transporters associated with *lpa* mutants, compared to those of other Arabidopsis ABCCs, put in evidence some peculiarities, the most significant being a very conserved stretch of lysine residues (consensus K/RXIKEKKKX$_{4\text{-}5}$R/KKK, yellow rectangle in Fig. 1), located in the cytosolic loop linking NBD1 to TMD2. Moreover, a number of charged amino acid residues (mostly Lys and Arg)

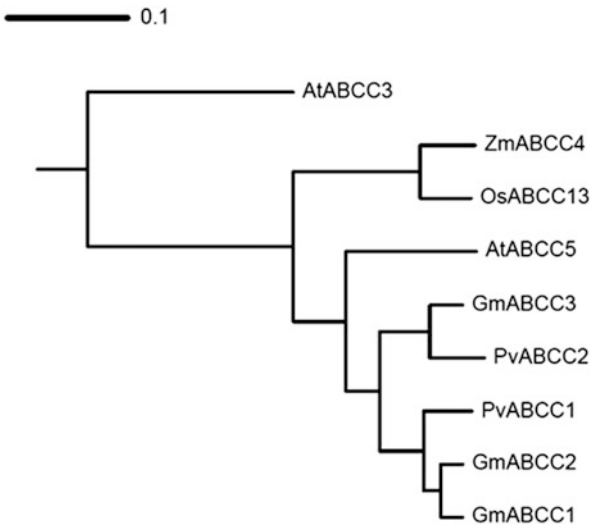

Fig. 2 Phylogenetic relationships among known MRP-type ABC phytic acid transporters associated to *lpa* mutations. The tree has been built comparing amino acid sequences of AtABCC5 (AtMRP5, Q7GB25); OsABCC13 (OsMRP5, NP_001048934); ZmABCC4 (ZmMPR4, ABS81429); GmABCC1 (GmMRP1, XP_003521316); GmABCC2 (GmMRP2 XP_003554305), GmABCC3 (GmMRP3, XP_003541373); PvABCC1 (PvMRP1, CBX25010); PvABCC2 (PvMRP2, CBX25011). The AtABCC3 (AtMRP3, Q9LK64) sequence not coding for a phytic acid transporter has been used as outgroup

are found in other conserved stretches located in TMD1 and TMD2 domains that might play a role in phytic acid transport.

Phylogenetic analyses of MRP type transporters indicate that $InsP_6$ transporters are represented by single copy genes (Klein et al. 2006; Wanke and Kolukisaoglu 2010; Kang et al. 2011); however it has been recently shown that soybean and common bean, two closely related legume species, bear a paralog copy (PvABCC2/PvMRP2 and GmABCC3/GmMRP3 in Fig. 2) (Panzeri et al. 2011).

Transgenic plants harboring the *GUS* reporter gene under the control of the *AtMRP5* promoter showed GUS staining mainly in vascular tissues of cotyledons, leaves (with the exception of xylem cells), roots (in the central cylinder, not in the root cortex and in the root tip) and anthers, in epidermal cells, particularly in guard cells and at the silique attachment site of the pedicel (Gaedeke et al. 2001). In the paper from Gaedeke and colleagues (2001) no data are available concerning GUS activity in the seeds. However from publicly available microarray data, it is clear that *AtMRP5* is expressed at different stages of seed development, particularly, from the pre-globular to the linear cotyledon stage it is mainly expressed at the chalazal seed coat, while at the maturation green stage it is very highly expressed in the entire seed, particularly in the embryo (http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi, Winter et al. 2007). Analysis of *AtMRP5* orthologs revealed a

broad expression pattern both in developing seeds and in different plant organs (Shi et al. 2007; Xu et al. 2009; Panzeri et al. 2011; Patel et al. 2012).

In seeds, $InsP_6$ is normally sequestered in structures called globoids in specialized vacuoles, termed protein storage vacuoles (Lin et al. 2005; Ockenden et al. 2004; Regvar et al. 2011; Krishnan 2008), then tonoplast subcellular localization for this family of transporters should be expected. Proteomic analysis predicts AtMRP5 to reside in the vacuolar membrane (Jaquinod et al. 2007) and the AtMRP5–GFP fusion protein, expressed under the control of the *AtMRP5* native promoter, gives a weak fluorescence signal associated with the membrane of vacuoles, released by osmotic lysis from leaf protoplasts, isolated from plants subjected to 14 days of drought stress (Nagy et al. 2009). In previous analysis, the same authors found that a functional AtMRP5–GFP fusion protein under the control of the constitutive *CaMV35S* promoter, expressed in transgenic *mrp5-1* mutant plants, was clearly targeted to the plasma membrane of root cells (Suh et al. 2007) and they suggested it could be an ectopic consequence of AtMRP5 overexpression. However, it is not clear if a GFP signal in the plasma membrane is present also in plants harboring the fusion protein under the control of the *AtMRP5* promoter. A possible dual localization was reported for other AtABCC proteins (Geisler et al. 2004; Klein et al. 2006; Rea 2007) and at the current state it cannot be excluded for AtMRP5.

3 Low Phytic Acid Mutants in MRP-Type ABC Phytic Acid Transporters

As already mentioned, different *low phytic acid* (*lpa*) mutants have been isolated in main crops, such as maize, barley, rice, wheat, soybean, and common bean (Raboy 2009). The *lpa* mutations can be classified into three categories, depending on the step of the biosynthetic pathway or transport they affect (Fig. 3): (1) mutations affecting the first steps of the biosynthetic pathway (from glucose 6-P to *myo*-inositol[3]-monophosphate); (2) mutations perturbing the end of the $InsP_6$ pathway (from *myo*-inositol[3]-monophosphate to $InsP_6$); (3) mutations affecting the transport of $InsP_6$. Mutants belonging to the first and the third categories are generally characterized by decreased $InsP_6$ levels, accompanied by a molar equivalent increase in inorganic P_i, but not by accumulation of lower $InsP_s$ (inositol phosphates with up to five phosphate residues), a characteristic specific for the second class of mutants. Moreover the two classes of mutants share a general perturbation of the different branches of the $InsP_6$ and of other compounds (galactinol, raffinose, stachyose, ascorbic acid) biosynthetic pathways. These similarities between the two classes of mutants triggered some confusion in the previous characterization of some of the mutants in the transporter genes. The initial hypothesis was that these mutants were affected in the *myo*-inositol 3-phosphate synthase (*MIPS*) gene, coding for the first enzyme of the pathway (Raboy et al. 2000; Pilu et al. 2003).

This confusion was also supported by genomic and expression data, since in maize the *ZmMIPS1S* and the *ZmMRP4* genes map very closely on chromosome 1S, and in mutants affecting *ZmMRP4* the expression of *ZmMIPS1S* is reduced (Raboy et al. 2000; Pilu et al. 2003; Shukla et al. 2004; Shi et al. 2007). However, mapping of the locus for low phytic acid and high inorganic phosphate content in the kernels of a specific maize line led to the identification of *ZmMRP4* (Shi et al. 2007). Phylogenetic studies identified the AtMRP5/AtABCC5 as the closest Arabidopsis homologue of ZmMRP4 (Nagy et al. 2009). Later, *lpa* mutations already isolated in rice, soybean, and common bean (Liu et al. 2007; Campion et al. 2009; Wilcox et al. 2000) were mapped in genes orthologous to *ZmMRP4* and *AtMRP5* (Xu et al. 2009; Maroof et al. 2009; Gillman et al. 2009, 2013; Panzeri et al. 2011). Curiously, the AtMRP5 transporter was under investigation since a decade as a transporter involved in glutathione conjugate and glucuronide conjugate transport activity. In the course of those years, several phenotypes were described for the *mrp5* mutant that apparently were not connected to the abolished ability to store phytic acid in the vacuole. The mutant showed reduced root growth, increased lateral root formation, affected regulation of stomata movement, modified guard cell hormonal signaling, and improved water use efficiency (Gaedeke et al. 2001; Klein et al. 2003; Suh et al. 2007).

As reported in general for *lpa* mutants, also mutants affected in genes coding for MRP type $InsP_6$ transporters may display negative pleiotropic effects on plant and seed performance, such as low germination rates, reduced seed development and weight, stunted vegetative growth, making these mutants of a limited value to breeders (Raboy et al. 2000; Meis et al. 2003; Pilu et al. 2005; Bregitzer and Raboy 2006; Guttieri et al. 2006). However, these pleiotropic effects differ from one species to another and from the different sites of mutations in the same gene in the case of point mutations. Moreover, there are also some exceptions, in fact some of these mutations do not cause these negative effects, such as knock out in the Arabidopsis *AtMRP5* gene that, conversely, confers increased tolerance to drought (Klein et al. 2003), and a mutation in the common bean *PvMRP1* (*PvABBC1*) gene that does not cause evident negative pleiotropic effects on traits relevant for agronomic performance (Campion et al. 2009, 2013). A description of the main characteristics of the Arabidopsis, cereals, and legume *MRP* genes and corresponding *lpa* mutants is presented in the following sections.

3.1 Arabidopsis

AtMRP5 gene was previously described for functions initially not correlated with its role in $InsP_6$ transport (Gaedeke et al. 2001; Klein et al. 2003; Lee et al. 2004; Suh et al. 2007) and, only when it was established its homology with *ZmMRP4*, a *lpa* phenotype was described in insertional mutants of this gene (Nagy et al. 2009). In fact, this protein was initially described for its important role in integrating several signals regulating stomatal movements.

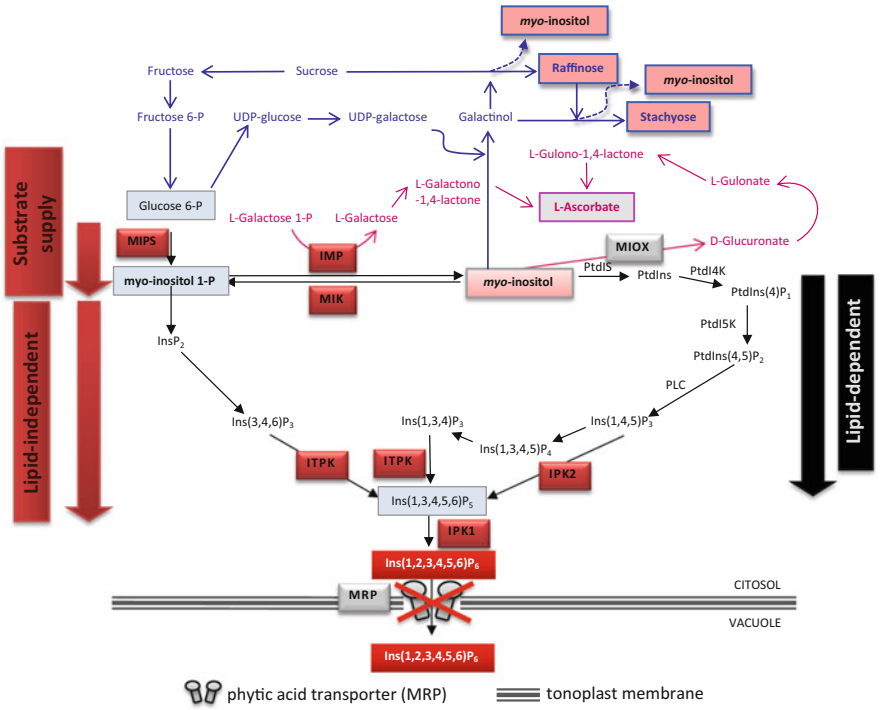

Fig. 3 Schematic representation of phytic acid biosynthetic pathway (*black*) and *myo*-inositol-derived pathways for ascorbic acid (*purple*) and raffinose-type oligosaccharides (*blue*). The substrate supply, lipid-independent (*red*) and lipid-dependent (*dark grey*) sub-pathways for $InsP_6$ synthesis are indicated. Metabolites and genes that have been reported to be downregulated in *lpa* (*mrp*) mutants are *red boxed*. *MIPS myo*-inositol-3-phosphate synthase, *IMP* bifunctional enzyme: *myo*-inositol-phosphate monophosphatase and galactose-1-phosphate phosphatase, *MIOX myo*-inositol monooxygenase, *MIK myo*-inositol kinase, *IPK2* inositol 1,4,5-tris-phosphate kinase, *ITPK* inositol 1,3,4-triphosphate 5/6-kinase, *IPK1* inositol 1,3,4,5,6 penta*kis*phosphate 2-kinase, *PtdIS* phosphatidyl inositol phosphate synthase, *PtdI4K* phosphatidyl inositol 4-kinase; *PtdI5K* phosphatidyl inositol 5-kinase, *PtdIns* phosphatidyl inositol; *PtdIns(4)P1* phosphatidyl inositol 4-phosphate; PtdIns(4,5)P2, phosphatidyl inositol 4,5-biphosphate, *PLC* phospholipase C

Initial work reported that *mrp5-1* mutant guard cells were no longer responsive to the sulfonylurea compound glibenclamide (Gaedeke et al. 2001; Suh et al. 2007), a well-known modulator of K-ATP channels and CFTR chloride channels, able to trigger stomatal opening in darkness in wild-type plants in a dose-dependent manner (Leonhardt et al. 1997). Further studies demonstrated that *mrp5-1* stomata opening was less sensitive to light. Moreover, *mrp5-1* guard cells did not respond to external calcium and to the phytohormone abscisic acid (ABA) that normally induce stomatal closure, and to auxin, that normally induces opening under darkness. However, mutant guard cells maintained normal response to fusicoccin promoting their opening in darkness and to elevated concentrations of CO_2 that induce stomatal closure (Klein et al. 2003). Defective stomatal responses were

relieved by guard cell targeted expression of AtMRP5 under the control of a guard cell-specific promoter (Nagy et al. 2009; Cominelli et al. 2011).

To a macroscopic level the guard cell phenotype of *mrp5-1* mutant confers reduced water loss from detached rosette leaves, reduced transpiration rate, improved water use efficiency, and enhanced drought stress resistance (Klein et al. 2003). Some of these effects were later explained by electrophysiological measurements demonstrating that the mutation impairs both ABA and cytosolic Ca^{2+} activation of slow (S-type) anion channels and ABA activation of Ca^{2+} permeable channel currents in the plasma membrane of guard cells (Suh et al. 2007). These data provide evidence that AtMRP5 is a central regulator of ion channels during ABA and Ca^{2+} signal transduction in guard cells. As former studies showed that $InsP_6$ is an activator of vacuolar Ca^{2+} release (Lemtiri-Chlieh et al. 2003) and an inhibitor of K^+ influx (Lemtiri-Chlieh et al. 2000), the role of vacuolar phytate transport mediated by AtMRP5 may be fundamental in this process.

The levels of $InsP_6$ in leaf tissue of Arabidopsis were not measured, because they were below the level of detection (Nagy et al. 2009), so at the moment it is not clear if there are some differences in the $InsP_6$ content between mutant and wild-type leaves and even more so between mutant and wild-type guard cells. However Nagy et al. (2009) proposed a hypothetical model that links AtMRP5 $InsP_6$ signaling and guard cell movements. In this model $InsP_6$ induces the release of Ca^{2+} from the vacuole to the cytosol and blocks K^+ flux from inward channels. AtMRP5 is necessary to transport $InsP_6$ from the cytosol to the vacuole thus avoiding the continuous $InsP_6$ signaling. Mutations in AtMRP5 affect $InsP_6$ export into the vacuole, causing an increase of $InsP_6$ concentration in the cytosol. Cytosolic $InsP_6$ might bind to Ca^{2+} and other divalent cations and/or may induce a continuous Ca^{2+} release, thus disturbing Ca^{2+}-dependent signaling pathway. Moreover, it may reduce K^+ uptake into guard cells by inhibiting K^+ inward rectifying channels.

Besides its role in the regulation of stomatal responses, AtMRP5 is also involved in other aspects related to the activity of $InsP_6$ in signaling. Particularly, *mrp5-1* seedlings grown on standard medium (0.5× MS, 1 % sucrose) showed a reduction in primary root elongation, accompanied by an earlier growth of lateral and secondary roots. On the contrary, when growth assays were performed on a more complete medium (1× MS medium added with different salts) in which *myo*-inositol was added, a reverse phenotype was obtained, suggesting that in the mutant *myo*-inositol levels are also reduced, as demonstrated for other mutants affecting $InsP_6$ transport in other species (Doria et al. 2009; Xu et al. 2009; Panzeri et al. 2011). The root phenotype on standard medium was explained by a twofold increase in auxin level in *mrp5-1* compared with the wild type (Gaedeke et al. 2001), indicating that $InsP_6$ transport is important for auxin accumulation and signaling. It is clear that AtMRP5 is also involved in different response to ABA, besides in guard cells, as the *mrp5-1* showed reduced sensitivity to ABA during germination (Klein et al. 2003). Moreover AtMRP5 has a quite complex role in response to abiotic stress as on one side *mrp5-1* is more drought tolerant, but on the

other side another knock out mutant showed hypersensitivity to Na^+ stress (Lee et al. 2004).

Concerning AtMRP5 role in phytic acid accumulation in seeds it was shown that *mrp5-1* and *mrp5-2* insertional mutants had a drastic *lpa* phenotype, as they do not accumulate $InsP_6$ in the seeds (Nagy et al. 2009). Moreover, they did not show increase in the level of lower inositol phosphates and displayed a reduction of more than 30 % in total phosphorus seed content. The lack of phytic acid accumulation caused a general repression of the biosynthetic pathway at gene expression level. In fact, expression of genes coding for key enzymes of the phytic acid pathway (*AtMIK*, *AtIPK2β*, *AtITPK1*, *AtITPK4*) was downregulated in developing siliques in *atmrp5-2* insertional mutant (Kim and Tai 2011). These data are in agreement with findings on other *mrp*-type *lpa* mutants of maize and common bean (Pilu et al. 2003, 2005; Panzeri et al. 2011). On the other side, *AtMRP5* gene expression was enhanced in developing siliques of most of the other *lpa* mutants (Kim and Tai 2011).

3.2 Cereals

Depending on the size of the grain, cereals preferentially accumulate phytic acid in different parts of the seeds. Small cereal grains, such as wheat, rice, and barley, store most of the phytic acid (up to 80 %) in the bran, the remaining being accumulated in the embryo. Conversely, in maize kernel the majority of the phytic acid, from 88 to 95 %, according to different authors, is present in the embryo, with the remainder in the aleurone layer (Schlemmer et al. 2009). Different mutations affecting the maize ZmMRP4 and the rice OsMRP5 phytic acid transporters have been isolated and characterized (Raboy et al. 2000; Pilu et al. 2003, 2005, 2009; Shukla et al. 2004; Lin et al. 2005; Shi et al. 2007; Liu et al. 2007; Doria et al. 2009; Xu et al. 2009; Cerino Badone et al. 2010; Landoni et al. 2013).

In maize three different mutants affected in the *ZmMRP4* locus were mainly characterized: *lpa1-1*, consisting in a point mutation that causes a A1432V substitution (Fig. 1) (Shi et al. 2007); *lpa1-241* mutation, a paramutagenic allele (Pilu et al. 2009) that causes a remarkable variability of expression with a different degree of negative pleiotropic effects depending on its strength (Pilu et al. 2005); *lpa1-7* mutation, whose molecular feature is not known, although the nature of a paramutagenic allele can be excluded (Cerino Badone et al. 2012). Mutants *lpa1-241* and *lpa1-7* showed the highest reduction in phytic acid content in the kernels with more than 85 % in the strongest lines of *lpa1-241* allele and about 80 % in the case of *lpa1-7* (Cerino Badone et al. 2012; Pilu et al. 2005). $InsP_6$ was reduced of 66 % in the *lpa1-1* mutant (Raboy et al. 2000). Consequently these mutants are characterized by a five- to tenfold increase in the amount of free phosphate, without alteration in the level of total phosphorous and of lower inositol phosphates (Pilu et al. 2003; Raboy et al. 2000; Cerino Badone et al. 2012).

Also in rice different mutant alleles at the *OsMRP5* locus have been identified (Liu et al. 2007). The *Os-XS-lpa2-1* mutant shows a seed $InsP_6$ reduction of about 20 % due to a single base pair substitution causing a P1156S change in the transmembrane domain TMD2 (Fig. 1) (Xu et al. 2009). On the contrary, in the *Os-XS-lpa2-2* mutant a 5-bp deletion occurred which determined a frame shift starting at position 452, causing a premature stop codon at aa 474. This loss of function is accompanied by a very high decrease in $InsP_6$ content (>90 %). Moreover a T-DNA knock out line (4A-02500) was isolated with a 90 % reduction in $InsP_6$ content (Xu et al. 2009).

It is interesting to observe that the highest reduction of $InsP_6$ content is strictly related to the strongest severity of the negative pleiotropic effects shown by these maize and rice *lpa* mutants. In fact the maize *lpa1-7* mutant, the strongest lines of the *lpa1-241* allele, the rice *Os-XS-lpa2-2* and 4A-02500 mutants are lethal as homozygous, although they could be rescued to some extent by embryo culture, while the other mutants (*lpa1-1* and *Os-XS-lpa2-1*) did not show such a severe phenotype (Raboy et al. 2000; Pilu et al. 2005; Xu et al. 2009; Cerino Badone et al. 2012). Histological analysis revealed that affected germination of the *lpa1-241* and *lpa1-7* mutants is probably due to profound alterations in the structure of the embryo, mainly the displacement of the root primordium from the embryonic axis that introduces an asymmetry into the body plan (Pilu et al. 2005; Cerino Badone et al. 2012).

Doria and colleagues (2009) proposed an explanation for the embryo lethality of these maize mutants. They showed that whole *lpa1-241* mutant kernels contained about 50 % more free or weakly bound iron than the wild type ones, expected to correlate with increase in iron bioavailability in term of nutritional importance. A higher content of free radicals, mainly concentrated in embryos, is associated with this aspect (Doria et al. 2009). To this purpose the *lpa1-241* mutant was used to show the function of phytic acid in the prevention of oxidative stress in seeds (Doria et al. 2009), previously only suggested (Graf et al. 1987; Graf and Eaton 1990; Empson et al. 1991) and considered to be important for the maintenance of the long-term viability of seeds (Raboy et al. 2000; Dorsch et al. 2003). In fact a higher production of hydrogen peroxide was found in the embryo scutellum of *lpa1-241* seeds with respect to the wild type ones, particularly in seeds artificially aged (incubated for 7 days in accelerating aging conditions consisting in 46 °C and 100 % relative humidity), a test normally used to evaluate susceptibility of seeds to storage. The *lpa1-241* mutation is then correlated to a higher degree of oxidative stress that seeds must cope with during maturation and ageing and that eventually results in loss of embryo viability. This pleiotropic effect of the mutation in the phytic acid transporter may be restricted to maize, where phytic acid is mainly accumulated in the embryo. In other species accumulating phytic acid in tissues different from the embryo, or endowed with other kinds or quantities of antioxidant compounds, a different behavior may be described, but specific data are still lacking (Doria et al. 2009). Ascorbic acid is a metabolite which synthesis is strictly related to phytic acid pathway (Fig. 2). Reduced content has been reported in Arabidopsis *vtc4 imp* double mutant, whereas a twofold increase in ascorbic acid have been

found in leaves of Arabidopsis plants ectopically expressing the *AtPAP15* gene, coding for a purple acid phosphatase with phytase activity (Zhang et al. 2008; Torabinejad et al. 2009). Since ascorbic acid is a strong antioxidant, it would be interesting to verify if any reduction occurs in *lpa-1-241* and *lpa1-7* seeds.

The strongest maize *lpa1* mutants, once rescued, show negative pleiotropic effects at seedling and adult plant stages, such as slow growth rate and stunted vegetative growth (Cerino Badone et al. 2012; Pilu et al. 2003, 2005). Alterations of the root phenotype for the *lpa1-7* mutant (Cerino Badone et al. 2012), similar to the ones described for the Arabidopsis *mrp5* mutant (Gaedeke et al. 2001), was also reported. In fact, *lpa1-7* plants failed to produce a functional primary root that stopped its elongation at an early stage, the development of secondary roots partially compensated for this lack (Cerino Badone et al. 2012). Moreover, as demonstrated for other *lpa* mutants affected in other genes, *lpa1-1* is more sensitive to drought stress in the field and this negative pleiotropic effect could be associated with an alteration of the mature root system, as demonstrated for *lpa1-7* (Cerino Badone et al. 2012).

On the other side, few negative pleiotropic effects were described for the weaker maize and rice mutants. In the case of the maize *lpa1-1* mutant only seed dry weight loss, ranging from 4 to 23 % was shown (Raboy et al. 2000). Different aspects of agronomic performance (grain weight and yield) and seed viability for the *Os-XS-lpa2-1* mutant were analyzed (Zhao et al. 2008). It was found a 5 % reduction of grain weight, while no significant differences in grain yield were highlighted. The analysis of seed viability and field emergence also showed the reduced performance of the *Os-XS-lpa2-1* mutant compared to the wild-type line. The simplified vigor index (calculated combining results from germination rates, seedling weight and seedling height) was 7.8 % less than that of the wt and field emergence rates were lower as well (about 65 % compared to 84 % of wt); however, when seeds were subjected to an artificial ageing test, the germination rates of *Os-XS-lpa2-1* seeds were not significantly different from those of wt (Cheng et al. 2007). The finding that the maize *lpa1-1* and the rice *Os-XS-lpa2-1* mutants were less severely affected than their respective more severe allelic mutants (*lpa1-7*, *lpa1-24*, *Os-XS-lpa2-2* and 4A-02500), may be partially due to residual $InsP_6$ transport activity, sufficient to allow the seedlings and the plant to cope, at least to some extent, with the environment.

Low phytic acid mutations can result in content changes of other seed constituents, such as *myo*-inositol, raffinose, and sucrose. Interestingly, when *myo*-inositol was measured in seeds of *Os-XS-lpa2-2*, or in the similar loss of function allele, 4A-02500, it was found that its content was much higher than that of the parent seeds with an increase of 89.2 % and 409 %, respectively (Xu et al. 2009). This was quite a surprising result since the other allelic mutant *Os-XS-lpa2-1*, which however shows a milder *lpa* phenotype, had significantly decreased content of *myo*-inositol ranging between 40 and 48 %. This finding, although in an opposite fashion, is very similar to what was reported for the allelic maize *lpa* mutants, where the milder *lpa1-1* allele has a 60 % of *myo*-inositol increase (Shi et al. 2007), whereas the stronger *lpa1-241* allele shows a decrease of 40 % (Doria et al. 2009).

Other pleiotropic effects of maize mutants were shown (Cerino Badone et al. 2010, 2012). It was observed that the *lpa1-241* mutation enhances the accumulation of anthocyanin in the kernel, changing the color of scutellum in the strongest *lpa1-241* mutant from dark red to bluish. This alteration was attributed to a defect in the pigment transport in the vacuole, causing a mislocalized accumulation of these pigments in the cytosol, suggesting that ZmMRP4 could have a direct or indirect role in anthocyanin transport. This phenotypic alteration is not present in plants harboring the weaker *lpa1-1* allele (Cerino Badone et al. 2010). In the *lpa1-7* mutant other effects referable to yet uncharacterized signaling function of $InsP_6$ are: reduced chlorophyll and carotenoid content and increased trichome density and length compared with sibling leaves (Cerino Badone et al. 2012).

To overcome the negative pleiotropic effects present in maize *lpa1* mutants, an alternative strategy to mutagenesis was undertaken, consisting in the tissue-specific silencing of the *ZmMRP4* gene. Embryo-specific expression of an antisense sequence for a fragment of the cDNA for the maize transporter produced *lpa*, high P_i transgenic maize seeds that germinate normally and do not have any significant reduction in seed dry weight (Shi et al. 2007), suggesting that probably the different pleiotropic effects depend on *ZmMRP4* expression pattern in different organs (Shi et al. 2007). A similar expression pattern in both vegetative tissues and developing seeds was shown for the *OsMRP5* gene (Xu et al. 2009).

3.3 Legumes

Legume seeds typically contain about 50 % or more seed total P than cereals and most of this P (about 85 %) is in the form of $InsP_6$, so in absolute terms the amount of $InsP_6$ in cereal seeds ranges between 2 and 3 mg/g of phytic acid phosphorus ($InsP_6$-P), while seeds of legume crops, such as soybean and common bean contain 4–5 mg/g of $InsP_6$-P. This means that the impact of developing *lpa* mutations in legume seeds is more relevant on a single seed basis, although it should be considered that in term of global production cereals are much higher than legumes.

Mutants in the MRP-type ABC phytic acid transporter have been identified both in soybean and common bean, two of the most relevant legume crops worldwide. Chronologically, the first *lpa* mutant lines, M153 and M766, were identified in soybean and it was shown that the *lpa* trait was controlled by two recessive alleles at two independent loci, named *pha1* and *pha2* (later renamed *lpa1* and *lpa2*, respectively). Only individuals homozygous for the two recessive alleles display the *lpa* trait and phytic acid is reduced by 80 % in M153 and 76.3 % in M766 seeds, respectively. However, higher $InsP_6$ reduction up to 94 % can be achieved combining the *lpa1* allele from M153 (*lpa1-a*) with the *lpa2* allele (*lpa2-b*) from M766 (Wilcox et al. 2000; Oltmans et al. 2004, 2005; Gillman et al. 2013). These two loci (*lpa1* and *lpa2*) were recently shown to correspond to two genes (*Glyma03g32500* and *Glyma19g35230*, respectively) coding for ABCC transporters (GmABCC1/GmMRP1 and GmABCC2/GmMRP2, respectively) homologous to ZmMRP4 and

AtMRP5 (Maroof et al. 2009; Gillman et al. 2009, 2013). The finding that the *lpa* trait is under the control of two genes is not surprising, since it is known that soybean underwent multiple polyploidization events (Shoemaker et al. 2006). Indeed, a comparative analysis of the genes involved in phytic acid synthesis between soybean and common bean revealed that in most cases for each bean gene two orthologous ones were found in the soybean genome (Fileppi et al. 2010).

The analysis of the molecular basis in the *lpa1* and *lpa2* alleles showed that the *lpa1-a* allele carries a nonsense mutation at R893, which results in a truncated protein (Fig. 1) (Maroof et al. 2009; Gillman et al. 2009). In the case of the M766 line (*lpa1-b* allele) a single T > A SNP 7 bp upstream of the start of exon 10 was identified. This SNP introduced an alternative splicing site, which resulted in five additional base pairs from the intron sequence and a frame shift starting at exon 10 (Fig. 1). Concerning the *lpa2* gene, the *lpa2-a* allele (M153) was found to carry a R1039K change, while in the *lpa2-b* allele a single base change causes a premature termination, again at position 1039 (Fig. 1) (Gillman et al. 2013).

Analysis of agronomic and seed related traits of soybean M455 *lpa* derived lines showed a decreased plant density (7.9 plants m^{-2} less than wt), a significant lower seedling emergence percentage (22–23 % less than wt) and an adverse effect on seed viability when seeds were reproduced in subtropical environment (Hulke et al. 2004; Anderson and Fehr 2008). However, genetic improvement through backcrossing was demonstrated to be successful to develop *lpa* lines with normal field emergence (Spear and Fehr 2007).

In common bean only one *lpa* mutant (*lpa1*, also known as *lpa280-10*) has been reported so far (Campion et al. 2009). The *lpa1* mutant is the result of a defective ABCC protein (Pvmrp1), belonging to the cluster grouping phytic acid transporters, in which a highly conserved Glu changed to Lys at a position 1155, in the transmembrane domain TMD2 (Fig. 1). The *lpa1* seeds display a very strong phenotype, consisting in a 90 % reduction of phytic acid accumulation, together with a decrease of raffinose containing sugars by 25 % and *myo*-inositol by 30 % (Campion et al. 2009; Panzeri et al. 2011). Agronomic analyses of the original *lpa1* mutant and of derived *lpa1* lines showed that, despite the strong $InsP_6$ reduction in the seed, seedling emergence, seed yield, and plant growth were not statistically different from those of wt and parental genotypes (Campion et al. 2009, 2013). To further verify the ability of *lpa1* seeds to survive stressful germination conditions, seed accelerated ageing test (AAT) and stress integrated germination test (SIGT) were carried out. Surprisingly, the *lpa1* seeds performed equally (SIGT) or even better (AAT) than the wt ones (Campion et al. 2009). A possible explanation for this finding is that in common bean a second gene, *PvMRP2*, paralog of *PvMRP1*, is present and it is most likely able to complement the absence of a functional *PvMRP1* in other tissues and organs than the seed. In soybean, a paralog (*Glyma13g18960*) to the *lpa1* and *lpa2* alleles (*Glyma03g32500* and *Glyma19g35230*) also exists (Panzeri et al. 2011) (Fig. 2). Expression analyses by qRT-PCR confirm that *PvMRP2* is expressed in almost all the tissues in a similar fashion to *PvMRP1*, with the exception of developing seeds (Cominelli et al., unpublished results) and a similar behavior is found also for the corresponding

soybean genes (http://bar.utoronto.ca/efpsoybean/cgi-bin/efpWeb.cgi) (Patel et al. 2012).

Klein and coworkers reported that the Arabidopsis *mrp5* mutant has reduced sensitivity to ABA during germination (Klein et al. 2003). When bean *lpa1* seeds were germinated in the presence of ABA, their germination resulted to be hypersensitive to the presence of this phytohormone. The sensitivity of seed germination to ABA has been shown to correlate negatively to seed *myo*-inositol content (Zhang et al. 2008; Torabinejad et al. 2009; Donahue et al. 2010; Panzeri et al. 2011).

4 MRP-Type ABC Phytic Acid Transporters and $InsP_6$ Metabolism

The phytic acid biosynthetic pathway is quite complex and involves genes that might undergo different temporal and spatial expression in different crops, and gene redundancy might further complicate this picture. In particular, the localization of MIPS protein does not appear to coincide with $InsP_6$ accumulation sites. In Arabidopsis and other dicots seeds, the site of initial synthesis of inositol phosphates differs from the site of eventual accumulation of $InsP_6$ (Reddy and Sathe 2001). Accordingly, the MIPS protein is expressed at very early stages of seed development in maternal tissues from which then *myo*-insitol is supposed to be transported to the embryo (Arabidopsis and *Brassica napus*) or to the cotyledons (common bean and soybean) where it is used for the synthesis of phosphatidylinositols first and phytic acid and other minor derivatives later (Mitsuhashi et al. 2008; Dong et al. 2013). In monocots $InsP_6$ was shown to accumulate in both the embryo and aleurone layer, and in developing rice seeds *MIPS* expression is firstly observed in the scutellum and embryo and only later it appears in the aleurone layer (Yoshida et al. 1999).

The most striking feature shared by *lpa* mutants affected in genes coding for MRP type ABC $InsP_6$ transporters is the reduced expression of *MIPS*, and, where reported, of genes coding for other enzymes involved in $InsP_6$ biosynthesis (*IPK1*, *IPK2*, *ITPK*, *IMP*, *MIK*) (Fig. 3) (Shukla et al. 2004; Pilu et al. 2005; Nagy et al. 2009; Panzeri et al. 2011; Kim and Tai 2011). Together with this misregulation, altered levels of *myo*-inositol and some of its derivatives (raffinose-type oligosaccharides and galactose) were reported (Shi et al. 2007; Frank et al. 2007; Xu et al. 2009; Campion et al. 2009; Doria et al. 2009; Panzeri et al. 2011).

The phytic acid transporter is necessary to store phytate into the vacuoles of developing seeds as well as to remove excess of $InsP_6$ in non-storage organs, such as guard cells where $InsP_6$ has important regulatory roles (Lemtiri-Chlieh et al. 2003). Since the plant cell cannot accumulate and/or manage large amounts of phytate salts in the cytosol, the excesses of cytosolic phytate should be partially controlled by degradative processes, mediated by phosphatases and phytases. This

has been found to occur in common bean *lpa1* mutant, in which it has been hypothesised that a temporary increase of *myo*-inositol and/or lower InsPs, produced during seed development, most likely activates a negative feedback regulation of genes necessary for $InsP_6$ synthesis (Panzeri et al. 2011). A similar feedback regulation, caused by an excess of *myo*-inositol, has been reported in *Catharanthus roseus* cell cultures, where the addition of *myo*-inositol to the medium reduces MIPS and IPK2 protein levels (Mitsuhashi et al. 2005). Moreover, recently, it has been shown that MIPS protein is necessary for its own gene expression regulation through a mechanism based on the inhibition of heterochromatin spreading (Latrasse et al. 2013). These authors also provide evidence that *MIPS* down-regulation induced by flg22 treatment results in accumulation of heterochromatin marks on *MIPS1* promoter and release of MIPS1 from its own promoter. It might be hypothesized that a similar mechanism occurs in *mrp* mutants. Feeding *myo*-inositol to *mips1* plants did not restore the activity of an ectopically expressed *MIPS1* promoter, suggesting that the MIPS1 protein itself, and not its catalytic activity, is required for its regulation.

MIPS catalyzes the rate-limiting step in inositol biosynthesis, thus it may be important in determining the pool size of *myo*-inositol and its derivatives in cells. Moreover, *myo*-inositol can also be recycled from inositol containing compounds, and it is thus not clear whether de novo inositol synthesis plays a regulatory role in modulating phosphoinositide levels in multicellular organisms.

These findings may partially explain the fact that many phenotypes, such as reduced root growth, embryo development defects, ABA germination sensitivity, stunted seedling growth, observed in *lpa* (*mrp*) mutants have also been reported in *mips* mutants (Chen and Xiong 2010; Donahue et al. 2010; Luo et al. 2011). In Arabidopsis *mips* mutants defects related to proper embryo development and early seedling growth are most likely due to a reduced synthesis of PtdIns, which would cause membrane disorganization and eventually impact on auxin polar transport (Luo et al. 2011). A similar mechanism may be hypothesised in *lpa* (*mrp*) mutants as well and the level of *MIPS* reduction, caused by inability to transport $InsP_6$, is expected to be directly correlated to the severity of the embryo and seedling phenotype (Chen and Xiong 2010). Indeed, in maize *lpa1-241* and *lpa1-7* mutants the embryo (displacement of the scutellum and altered symmetry) and seedling growth defects are strictly correlated to a high decrease of phytic acid in the seed, as no such phenotypes are found in the *lpa1-1* mutant. Similarly, in Arabidopsis, embryo defects are detected only in double *mips* mutants (*mips1 mips2*$^{+/-}$ or *mips1 mips3*), confirming that de novo biosynthesis of inositol is essential for normal embryo development and seedling growth (Chen and Xiong 2010).

There are some exceptions to this observation: the *mrp5* and the *Pvmrp1* mutants do not display severe pleiotropic phenotypes related to seed development and seedling growth, although the seed $InsP_6$ reduction is very consistent. Most likely, the presence of multiple *MIPS* copies, which are coexpressed during seed development, may attenuate the negative feedback caused by the *mrp5* mutation. In common been paralogous *MRP* gene (*PvMRP2*) exists and is expressed at very similar levels to *PvMRP1* in all the plant, with the exception of developing seeds,

thus its presence may compensate for the lack of a functional copy in the plant. At the seed level, the largest amount of phytic acid is stored in the cotyledons, thus the effects of MIPS reduction are not expected to significantly affect the embryo development.

5 Conclusion

Failure of $InsP_6$ transport in *lpa* mutants causes perturbations of Ins and Ins phosphate metabolism, thus the pleiotropic effects reflect the relevance of $InsP_6$, inositol phosphates and their key precursor, *myo*-inositol, in fundamental signaling and developmental pathways (Raboy 2009). Indeed, there is an increasing literature showing that phytic acid and inositol polyphosphates play key roles in the regulation of several plant functions, like root architecture, nutrient uptake, stomata opening, ABA and stress signal transduction, pathogen response, etc. (Gunesekera et al. 2007; Stevenson-Paulik et al. 2005; Lemtiri-Chlieh et al. 2003; Xiong et al. 2001). Moreover, it has been found that $InsP_6$ and inositol penta*kis*phosphate ($InsP_5$) are structural cofactors for two structurally related F-box type proteins involved in the perception of auxin (transport inhibiting response 1, TIR1) and of jasmonic acid (isoleucin conjugate of jasmonic acid, COI1), respectively (Tan et al. 2007; Mosblech et al. 2011). Taken together these findings indicate that phytic acid in the plant cell is much more than a storage molecule, and its biosynthetic pathway and correct compartmentalization (dependent on MRP-type ABC transporters) are pivotal to fulfill many regulatory processes occurring during plant development and interaction with the environment.

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References

Anderson B, Fehr W (2008) Seed source affects field emergence of low-phytate soybean lines. Crop Sci 48(3):929–932

Bandler PE, Westlake CJ, Grant CE, Cole SP, Deeley RG (2008) Identification of regions required for apical membrane localization of human multidrug resistance protein 2. Mol Pharmacol 74(1):9–19

Bregitzer P, Raboy V (2006) Effects of four independent low-phytate mutations on barley agronomic performance. Crop Sci 46(3):1318–1322

Campion B, Sparvoli F, Doria E, Tagliabue G, Galasso I, Fileppi M, Bollini R, Nielsen E (2009) Isolation and characterisation of an lpa (low phytic acid) mutant in common bean (Phaseolus vulgaris L.). Theor Appl Genet 118(6):1211–1221

Campion B, Glahn R, Tava A, Perrone D, Doria E, Sparvoli F, Cecotti R, Dani V, Nielsen E (2013) Genetic reduction of antinutrients in common bean (Phaseolus vulgaris L.) seed, increases nutrients and in vitro iron bioavailability without depressing main agronomic traits. Field Crop Res 141:27–37

Cerino Badone F, Cassani E, Landoni M, Doria E, Panzeri D, Lago C, Mesiti F, Nielsen E, Pilu R (2010) The low phytic acid1-241 (lpa1-241) maize mutation alters the accumulation of anthocyanin pigment in the kernel. Planta 231(5):1189–1199

Cerino Badone F, Amelotti M, Cassani E, Pilu R (2012) Study of low phytic acid1-7 (lpa1-7), a new ZmMRP4 mutation in maize. J Hered 103(4):598–605

Chen H, Xiong L (2010) myo-Inositol-1-phosphate synthase is required for polar auxin transport and organ development. J Biol Chem 285(31):24238–24247

Cheng F, Liu Y, Liu Z, Zhao N, Wang F, Zhang Q, Zhang G (2007) Positional variations in chalky occurrence within a rice panicle and its relation to grain nutritional quality. Aust J Agr Res 58(2):95–103

Cominelli E, Galbiati M, Albertini A, Fornara F, Conti L, Coupland G, Tonelli C (2011) DOF-binding sites additively contribute to guard cell-specificity of AtMYB60 promoter. BMC Plant Biol 11(1):162

Donahue J, Alford S, Torabinejad J, Kerwin R, Nourbakhsh A, Ray W, Hernick M, Huang X, Lyons B, Hein P, Gillaspy G (2010) The Arabidopsis thaliana myo-inositol 1-phosphate synthase1 gene is required for myo-inositol synthesis and suppression of cell death. Plant Cell 22(3):888–903

Dong J, Yan W, Bock C, Nokhrina K, Keller W, Georges F (2013) Perturbing the metabolic dynamics of myo-inositol in developing Brassica napus seeds through in vivo methylation impacts its utilization as phytate precursor and affects downstream metabolic pathways. BMC Plant Biol 13:84

Doria E, Galleschi L, Calucci L, Pinzino C, Pilu R, Cassani E, Nielsen E (2009) Phytic acid prevents oxidative stress in seeds: evidence from a maize (Zea mays L.) low phytic acid mutant. J Exp Bot 60(3):967–978

Dorsch J, Cook A, Young K, Anderson J, Bauman A, Volkmann C, Murthy P, Raboy V (2003) Seed phosphorus and inositol phosphate phenotype of barley low phytic acid genotypes. Phytochemistry 62(5):691–706

Empson K, Labuza T, Graf E (1991) Phytic acid as food antioxidant. J Food Sci 56(2):560–563

Fileppi M, Galasso I, Tagliabue G, Daminati M, Campion B, Doria E, Sparvoli F (2010) Characterisation of structural genes involved in phytic acid biosynthesis in common bean (Phaseolus vulgaris L.). Mol Breed 25(3):453–470

Frank T, Meuleye B, Miller A, Shu Q, Engel K (2007) Metabolite profiling of two low phytic acid (lpa) rice mutants. J Agric Food Chem 55(26):11011–11019

Gaedeke N, Klein M, Kolukisaoglu U, Forestier C, Muller A, Ansorge M, Becker D, Mamnun Y, Kuchler K, Schulz B, Mueller-Roeber B, Martinoia E (2001) The Arabidopsis thaliana ABC transporter AtMRP5 controls root development and stomata movement. EMBO J 20(8): 1875–1887

Gao M, Yamazaki M, Loe D, Westlake C, Grant C, Cole S, Deeley R (1998) Multidrug resistance protein - Identification of regions required for active transport of leukotriene C-4. J Biol Chem 273(17):10733–10740

Geisler M, Girin M, Brandt S, Vincenzetti V, Plaza S, Paris N, Kobae Y, Maeshima M, Billion K, Kolukisaoglu U, Schulz B, Martinoia E (2004) Arabidopsis immunophilin-like TWD1 functionally interacts with vacuolar ABC transporters. Mol Biol Cell 15(7):3393–3405

Gillman J, Pantalone V, Bilyeu K (2009) The low phytic acid phenotype in soybean line CX1834 is due to mutations in two homologs of the maize low phytic acid gene. Plant Genome 2(2): 179–190

Gillman J, Baxter I, Bilyeu K (2013) Phosphorus partitioning of soybean lines containing different mutant alleles of two soybean seed-specific adenosine triphosphate-binding cassette phytic acid transporter paralogs. Plant Genome 6(1)

Graf E, Eaton J (1990) Antioxidant function of phytic acid. Free Radic Biol Med 8(1):61–69

Graf E, Empson K, Eaton J (1987) Phytic acid. A natural antioxidant. J Biol Chem 262(24): 11647–11650

Gunesekera B, Torabinejad J, Robinson J, Gillaspy GE (2007) Inositol polyphosphate 5-phosphatases 1 and 2 are required for regulating seedling growth. Plant Physiol 143(3):1408–1417

Guttieri MJ, Peterson KM, Souza EJ (2006) Agronomic performance of low phytic acid wheat. Crop Sci 46(6):2623–2629

Hulke B, Fehr W, Welke G (2004) Agronomic and seed characteristics of soybean with reduced phytate and palmitate. Crop Sci 44(6):2027–2031

Jaquinod M, Villiers F, Kieffer-Jaquinod S, Hugouvieu V, Bruley C, Garin J, Bourguignon J (2007) A proteomics dissection of Arabidopsis thaliana vacuoles isolated from cell culture. Mol Cell Proteomics 6(3):394–412

Kang J, Park J, Choi H, Burla B, Kretzschmar T, Lee Y, Martinoia E (2011) Plant ABC transporters. Arabidopsis Book 9:e0153

Kim S, Tai T (2011) Identification of genes necessary for wild-type levels of seed phytic acid in Arabidopsis thaliana using a reverse genetics approach. Mol Genet Genomics 286(2):119–133

Klein M, Perfus-Barbeoch L, Frelet A, Gaedeke N, Reinhardt D, Mueller-Roeber B, Martinoia E, Forestier C (2003) The plant multidrug resistance ABC transporter AtMRP5 is involved in guard cell hormonal signalling and water use. Plant J 33(1):119–129

Klein M, Burla B, Martinoia E (2006) The multidrug resistance-associated protein (MRP/ABCC) subfamily of ATP-binding cassette transporters in plants. FEBS Lett 580(4):1112–1122

Krishnan H (2008) Preparative procedures markedly influence the appearance and structural integrity of protein storage vacuoles in soybean seeds. J Agric Food Chem 56(9):2907–2912

Landoni M, Badone F, Haman N, Schiraldi A, Fessas D, Cesari V, Toschi I, Cremona R, Delogu C, Villa D, Cassani E, Pilu R (2013) Low phytic acid 1 mutation in maize modifies density, starch properties, cations, and fiber contents in the seed. J Agric Food Chem 61(19):4622–4630

Latrasse D, Jegu T, Meng P, Mazubert C, Hudik E, Delarue M, Charon C, Crespi M, Hirt H, Raynaud C, Bergounioux C, Benhamed M (2013) Dual function of MIPS1 as a metabolic enzyme and transcriptional regulator. Nucleic Acids Res 41(5):2907–2917

Lee E, Kwon M, Ko J, Yi H, Hwang M, Chang S, Cho M (2004) Binding of sulfonylurea by AtMRP5, an Arabidopsis multidrug resistance-related protein that functions in salt tolerance. Plant Physiol 134(1):528–538

Lemtiri-Chlieh F, MacRobbie E, Brearley C (2000) Inositol hexakisphosphate is a physiological signal regulating the K+-inward rectifying conductance in guard cells. Proc Natl Acad Sci USA 97(15):8687–8692

Lemtiri-Chlieh F, MacRobbie E, Webb A, Manison N, Brownlee C, Skepper J, Chen J, Prestwich G, Brearley C (2003) Inositol hexakisphosphate mobilizes an endomembrane store of calcium in guard cells. Proc Natl Acad Sci USA 100(17):10091–10095

Leonhardt N, Marin E, Vavasseur A, Forestier C (1997) Evidence for the existence of a sulfonylurea-receptor-like protein in plants: modulation of stomatal movements and guard cell potassium channels by sulfonylureas and potassium channel openers. Proc Natl Acad Sci USA 94(25):14156–14161

Lin L, Ockenden I, Lott J (2005) The concentrations and distribution of phytic acid-phosphorus and other mineral nutrients in wild-type and low phytic acid1-1 (lpa1-1) corn (Zea mays L.) grains and grain parts. Can J Bot-Revue Canadienne De Botanique 83(1):131–141

Liu Q, Xu X, Ren X, Fu H, Wu D, Shu Q (2007) Generation and characterization of low phytic acid germplasm in rice (Oryza sativa L.). Theor Appl Genet 114(5):803–814

Luo Y, Qin G, Zhang J, Liang Y, Song Y, Zhao M, Tsuge T, Aoyama T, Liu J, Gu H, Qu L (2011) D-myo-inositol-3-phosphate affects phosphatidylinositol-mediated endomembrane function in Arabidopsis and is essential for auxin-regulated embryogenesis. Plant Cell 23(4):1352–1372

Maroof M, Glover N, Biyashev R, Buss G, Grabau E (2009) Genetic basis of the low-phytate trait in the soybean line CX1834. Crop Sci 49(1):69–76

Mason DL, Michaelis S (2002) Requirement of the N-terminal extension for vacuolar trafficking and transport activity of yeast Ycf1p, an ATP-binding cassette transporter. Mol Biol Cell 13(12):4443–55

Meis SJ, Fehr WR, Schnebly SR (2003) Seed source effect on field emergence of soybean lines with reduced phytate and raffinose saccharides. Crop Sci 43(4):1336–1339

Mitsuhashi N, Ohnishi M, Sekiguchi Y, Kwon Y, Chang Y, Chung S, Inoue Y, Reid R, Yagisawa H, Mimura T (2005) Phytic acid synthesis and vacuolar accumulation in suspension-cultured cells of Catharanthus roseus induced by high concentration of inorganic phosphate and cations. Plant Physiol 138(3):1607–1614

Mitsuhashi N, Kondo M, Nakaune S, Ohnishi M, Hayashi M, Hara-Nishimura I, Richardson A, Fukaki H, Nishimura M, Mimura T (2008) Localization of myo-inositol-1-phosphate synthase to the endosperm in developing seeds of Arabidopsis. J Exp Bot 59(11):3069–3076

Mosblech A, Thurow C, Gatz C, Feussner I, Heilmann I (2011) Jasmonic acid perception by COI1 involves inositol polyphosphates in Arabidopsis thaliana. Plant J 65(6):949–57

Nagy R, Grob H, Weder B, Green P, Klein M, Frelet-Barrand A, Schjoerring J, Brearley C, Martinoia E (2009) The Arabidopsis ATP-binding cassette protein AtMRP5/AtABCC5 is a high affinity inositol hexakisphosphate transporter involved in guard cell signaling and phytate storage. J Biol Chem 284(48):33614–33622

Ockenden I, Dorsch J, Reid M, Lin L, Grant L, Raboy V, Lott J (2004) Characterization of the storage of phosphorus, inositol phosphate and cations in grain tissues of four barley (Hordeum vulgare L.) low phytic acid genotypes. Plant Sci 167(5):1131–1142

Oltmans S, Fehr W, Welke G, Cianzio S (2004) Inheritance of low-phytate phosphorus in soybean. Crop Sci 44(2):433–435

Oltmans S, Fehr W, Welke G, Raboy V, Peterson K (2005) Agronomic and seed traits of soybean lines with low-phytate phosphorus. Crop Sci 45(2):593–598

Panzeri D, Cassani E, Doria E, Tagliabue G, Forti L, Campion B, Bollini R, Brearley CA, Pilu R, Nielsen E, Sparvoli F (2011) A defective ABC transporter of the MRP family, responsible for the bean lpa1 mutation, affects the regulation of the phytic acid pathway, reduces seed myo-inositol and alters ABA sensitivity. New Phytol 191(1):70–83

Patel R, Nahal H, Breit R, Provart N (2012) BAR expressolog identification: expression profile similarity ranking of homologous genes in plant species. Plant J 71(6):1038–1050

Pilu R, Panzeri D, Gavazzi G, Rasmussen S, Consonni G, Nielsen E (2003) Phenotypic, genetic and molecular characterization of a maize low phytic acid mutant (lpa241). Theor Appl Genet 107(6):980–987

Pilu R, Landoni M, Cassani E, Doria E, Nielsen E (2005) The maize *lpa241* mutation causes a remarkable variability of expression and some pleiotropic effects. Crop Sci 45(5):2096–2105

Pilu R, Panzeri D, Cassani E, Cerino Badone F, Landoni M, Nielsen E (2009) A paramutation phenomenon is involved in the genetics of maize low phytic acid1-241 (lpa1-241) trait. Heredity 102(3):236–245

Raboy V (2001) Seeds for a better future: 'low phytate', grains help to overcome malnutrition and reduce pollution. Trends Plant Sci 6(10):458–462

Raboy V (2003) myo-Inositol-1,2,3,4,5,6-hexakisphosphate. Phytochemistry 64(6):1033–1043

Raboy V (2009) Approaches and challenges to engineering seed phytate and total phosphorus. Plant Sci 177(4):281–296

Raboy V, Gerbasi P, Young K, Stoneberg S, Pickett S, Bauman A, Murthy P, Sheridan W, Ertl D (2000) Origin and seed phenotype of maize low phytic acid 1-1 and low phytic acid 2-1. Plant Physiol 124(1):355–368

Raboy V, Young K, Dorsch J, Cook A (2001) Genetics and breeding of seed phosphorus and phytic acid. J Plant Physiol 158(4):489–497

Rea P (2007) Plant ATP-Binding cassette transporters. Annu Rev Plant Biol 58:347–375

Reddy N, Sathe S (2001) Food phytates, technology and engineering. CRC Press, Boco Raton, FL

Regvar M, Eichert D, Kaulich B, Gianoncelli A, Pongrac P, Vogel-Mikus K, Kreft I (2011) New insights into globoids of protein storage vacuoles in wheat aleurone using synchrotron soft X-ray microscopy. J Exp Bot 62(11):3929–3939

Schlemmer U, Frølich W, Prieto RM, Grases F (2009) Phytate in foods and significance for humans: food sources, intake, processing, bioavailability, protective role and analysis. Mol Nutr Food Res 53(Suppl 2):S330–75

Shi J, Wang H, Schellin K, Li B, Faller M, Stoop J, Meeley R, Ertl D, Ranch J, Glassman K (2007) Embryo-specific silencing of a transporter reduces phytic acid content of maize and soybean seeds. Nat Biotechnol 25(8):930–937

Shoemaker R, Schlueter J, Doyle J (2006) Paleopolyploidy and gene duplication in soybean and other legumes. Curr Opin Plant Biol 9(2):104–109

Shukla S, VanToai T, Pratt R (2004) Expression and nucleotide sequence of an INS (3) P-1 synthase gene associated with low-phytate kernels in maize (Zea mays L.). J Agric Food Chem 52(14):4565–4570

Spear J, Fehr W (2007) Genetic improvement of seedling emergence of soybean lines with low phytate. Crop Sci 47(4):1354–1360

Stevenson-Paulik J, Bastidas RJ, Chiou ST, Frye RA, York JD (2005) Generation of phytate-free seeds in Arabidopsis through disruption of inositol polyphosphate kinases. Proc Natl Acad Sci USA 102(35):12612–12617

Suh SJ, Wang YF, Frelet A, Leonhardt N, Klein M, Forestier C, Mueller-Roeber B, Cho MH, Martinoia E, Schroeder JI (2007) The ATP binding cassette transporter AtMRP5 modulates anion and calcium channel activities in Arabidopsis guard cells. J Biol Chem 282(3):1916–24

Tan X, Calderon-Villalobos LI, Sharon M, Zheng C, Robinson CV, Estelle M, Zheng N (2007) Mechanism of auxin perception by the TIR1 ubiquitin ligase. Nature 446(7136):640–5

Torabinejad J, Donahue J, Gunesekera B, Allen-Daniels M, Gillaspy G (2009) VTC4 Is a bifunctional enzyme that affects myoinositol and ascorbate biosynthesis in plants. Plant Physiol 150(2):951–961

Verrier PJ, Bird D, Burla B, Dassa E, Forestier C, Geisler M, Klein M, Kolukisaoglu U, Lee Y, Martinoia E, Murphy A, Rea PA, Samuels L, Schulz B, Spalding EJ, Yazaki K, Theodoulou FL (2008) Plant ABC proteins–a unified nomenclature and updated inventory. Trends Plant Sci 13(4):151–9

Wanke D, Kolukisaoglu H (2010) An update on the ABCC transporter family in plants: many genes, many proteins, but how many functions? Plant Biol 12:15–25

Westlake CJ, Cole SP, Deeley RG (2005) Role of the NH2-terminal membrane spanning domain of multidrug resistance protein 1/ABCC1 in protein processing and trafficking. Mol Biol Cell 16(5):2483–92

Wilcox J, Premachandra G, Young K, Raboy V (2000) Isolation of high seed inorganic P, low-phytate soybean mutants. Crop Sci 40(6):1601–1605

Winter D, Vinegar B, Nahal H, Ammar R, Wilson G, Provart N (2007) An "Electronic Fluorescent Pictograph" browser for exploring and analyzing large-scale biological data sets. Plos One 2(8):e718

Xiong L, Lee B, Ishitani M, Lee H, Zhang C, Zhu JK (2001) FIERY1 encoding an inositol polyphosphate 1-phosphatase is a negative regulator of abscisic acid and stress signaling in Arabidopsis. Genes Dev 15(15):1971–1984

Xu X, Zhao H, Liu Q, Frank T, Engel K, An G, Shu Q (2009) Mutations of the multi-drug resistance-associated protein ABC transporter gene 5 result in reduction of phytic acid in rice seeds. Theor Appl Genet 119(1):75–83

Yoshida K, Wada T, Koyama H, Mizobuchi-Fukuoka R, Naito S (1999) Temporal and spatial patterns of accumulation of the transcript of myo-inositol-1-phosphate synthase and phytin-containing particles during seed development in rice. Plant Physiol 119(1):65–72

Zhang W, Gruszewski H, Chevone B, Nessler C (2008) An Arabidopsis purple acid phosphatase with phytase activity increases foliar ascorbate. Plant Physiol 146(2):431–440

Zhao H, Liu Q, Fu H, Xu X, Wu D, Shu Q (2008) Effect of non-lethal low phytic acid mutations on grain yield and seed viability in rice. Field Crop Res 108(3):206–211

ABA Transport by ABCG Transporter Proteins

Takashi Kuromori and Kazuo Shinozaki

Abstract The superfamily of ATP-binding cassette (ABC) transporters is one of the most abundant protein families in nature. These transporters are broadly conserved from prokaryotes to higher eukaryotes, and most of them transport various metabolites and signalling molecules in an ATP-dependent manner. Plant genomes in particular have large ABC families of more than one hundred genes, implying that some ABC transporters have important functions in plant-specific development or environmental responses. Hormones are typically regulated at the site of biosynthesis and during transport to their site of action. ABC transporters were recently reported to be involved in the transport of phytohormones, of which abscisic acid (ABA) is one of the best-studied and considered to be a plant stress hormone. This chapter describes ABC transporters whose functions are related to ABA transport and associated responses.

1 Introduction

Abscisic acid (ABA) is a phytohormone that plays an important role in a broad range of plant traits including adaptation to environmental conditions. ABA signalling in response to abiotic stress has been investigated vigorously in relation to physiological effects including regulation of stomatal aperture. Over the past decade, the molecular basis of intracellular ABA signalling has been revealed. Intracellular ABA signalling is triggered by a complex with three major components including cellular ABA receptors, PP2C-type protein phosphatases and SnRK2-type protein kinases (Cutler et al. 2010; Hubbard et al. 2010; Klingler

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et al. 2010; Raghavendra et al. 2010; Umezawa et al. 2010). In contrast, the mechanism underlying intercellular ABA responses remains largely unknown even though it is as important as intracellular signalling.

Whether ABA is synthesised in roots and then transported to shoots or is produced directly in shoot tissues as an early response to osmotic stress is still debated (Sauter et al. 2001; Christmann et al. 2007). Nevertheless, ABA transport is suggested to exist in plants because although ABA is biosynthesised and metabolised predominantly in vascular tissues, it is also involved in the stomatal responses of surface cell layers (Cheng et al. 2002; Koiwai et al. 2004; Okamoto et al. 2009). Using antisense RNA or antibodies that are specific for ABA biosynthesis enzymes in *Arabidopsis* such as NCED3, ABA2 and AAO3, leaf vascular parenchyma cells were shown to be the primary sites of ABA biosynthesis in both drought-stressed and unstressed plants (Cheng et al. 2002; Koiwai et al. 2004; Endo et al. 2008). Under dehydration stress conditions, the NCED3 promoter was shown to be active mainly in vascular tissues (Behnam et al. 2013). Based on these results, ABA was proposed to be transported from the vascular tissue to guard cells, leading to accumulation of ABA in stomata (Seo and Koshiba 2011).

In contrast, the transport system of another important phytohormone, indole-3-acetic acid (auxin), has been shown to include both cellular efflux and influx carriers (Petrásek and Friml 2009; Vanneste and Friml 2009). In current models, auxin migrates during polar transport to adjacent cells to regulate disproportionate cell growth (Friml and Palme 2002; Tanaka et al. 2006). Whether ABA is transported in a polar manner between cells by specific transporters similar to auxin is unclear (Benjamins and Scheres 2008). However, the stress hormone ABA is required for rapid signalling especially under stress conditions (Kim et al. 2010). For example, ABA promotes stomatal closure in guard cells to prevent transpiration under drought conditions (Leung and Giraudat 1998; Cutler et al. 2010; Kim et al. 2010). Consistent with this function, ABA may be transported with apoplastic water flow. If this was the case, the first step in ABA transport would be its export from the inside to the outside of the cells that synthesise it.

The necessity for ABA transporters that act across plasma membranes can also be inferred from the hindrance to ABA movement caused by an anion trap (Hartung et al. 1998; Taiz and Zeiger 2006). Because ABA is a weak acid (p*K*a 4.7) that is present mostly in the ionised form in the cytosol where the pH is approximately neutral, it does not move passively across the plasma membrane lipid bilayer (Hartung et al. 1998; Taiz and Zeiger 2006). A specific transporter may be required for ABA movement from the cell interior to the exterior to overcome the anion trap. Similarly, elevated apoplastic pH, for example in guard cells under stress conditions could necessitate the activity of an ABA importer for the cellular uptake of ionised ABA (Kang et al. 2010).

Different types of ABA transporters were recently reported (Kang et al. 2010; Kuromori et al. 2010; Kanno et al. 2012), with mutant analysis identifying two ABC transporters, AtABCG25 and AtABCG40. Biochemical characterisation showed that AtABCG25 mediated ABA export from the cell and that AtABCG40 mediated import into the cell (Kang et al. 2010; Kuromori et al. 2010). More

recently, ABA-importing transporters belonging to the NRT1/PTR family were identified using a modified yeast two-hybrid screen and shown to import ABA (Kanno et al. 2012). A summary of these transporters and a current model of ABA transport are described in this chapter.

2 AtABCG25

Mutants of *AtABCG25* were originally isolated using an *Ac*/*Ds* transposon-tagged mutant series in a genetic screen for mutants that exhibited abnormal ABA sensitivity during the early growth stage (Kuromori et al. 2010). *AtABCG25* expression was detected mainly in vascular tissues where genes encoding ABA biosynthesis enzymes are predominantly expressed. In addition, fluorescent protein-fused AtABCG25 proteins were localised to the plasma membrane in plant cells.

AtABCG25 was examined to determine whether it exhibited ABA export activity. Because of its instability making the detection of in vivo export activity very difficult, a vesicle transport assay was used to detect the intrinsic export function through the uptake of labelled molecules. In membrane vesicles derived from AtABCG25-expressing insect cells, AtABCG25 exhibited ATP-dependent ABA transport activity. Furthermore, leaf temperatures were higher in AtABCG25-overexpressing plants than in wild-type plants under nonstress conditions, implying an effect on stomatal regulation. These results suggested that AtABCG25 is an exporter of ABA through the plasma membrane and is involved in intercellular ABA signalling.

AtABCG25 belongs to a group of half-size ABC transporter genes in the AtABCG subfamily previously called the WBC subfamily (Verrier et al. 2008). Because half-size ABC transporters usually function as dimer complexes, considerable discussion has focused on whether they function as homodimers or heterodimers (Graf et al. 2003; Samuels et al. 2008). Based on the detection of ABA transport activity in insect cell membranes, AtABCG25 quite possibly functions as a homodimer. Whether AtABCG25 can also function as a heterodimer with other half-size ABC transporters remains unclear.

3 AtABCG40

The *Arabidopsis* ABC transporter *AtABCG40*, which was reported independently and simultaneously with *AtABCG25*, functions as an ABA-importer in plant cells (Kang et al. 2010). An *atabcg40* mutant was originally identified by testing seed germination and stomatal movement in mutant lines with knockout mutations in a cluster of full-size ABC transporter genes in the AtABCG subfamily, previously designated as the PDR subfamily (Verrier et al. 2008). Based on promoter–GUS analysis, *AtABCG40* was expressed broadly in the leaves of young plantlets and in

primary and lateral roots. The highest expression was found in guard cells. Fluorescent protein-fused AtABCG40 proteins expressed under its native promoter were localised to plasma membranes in *Arabidopsis* guard cells.

Stomatal closure in response to ABA was slower in a*tabcg40* mutant plants, and the upregulation of ABA-inducible genes was significantly delayed, resulting in reduced drought tolerance. ABA uptake increased in both yeast and tobacco BY2 cells expressing AtABCG40, whereas ABA uptake by protoplasts derived from *atabcg40* plants decreased relative to control cells. These results suggest that AtABCG40 mediates ABA uptake as an ABA importer for rapid responses to environmental stress.

4 AtABCG22

AtABCG22, another ABCG gene closely related to *AtABCG25*, is required for stomatal regulation (Kuromori et al. 2011). Rosette leaves of *atabcg22* mutant plants exhibited lower temperatures and increased water loss, resulting in greater susceptibility to drought stress than wild-type plants. *AtABCG22* is expressed in aerial organs primarily in guard cells, consistent with the mutant phenotypes. Fluorescent protein-fused AtABCG22 was localised to the plasma membrane in plant cells. Based on these results, AtABCG22 is considered to be a candidate ABA transporter, although ABA transport activity has not been demonstrated for this protein.

5 AtNPF4.6/NRT1.2/AIT1

More recently, ABA-importing transporters (AIT1–4) that belong to the NRT1/PTR transporter family rather than the ABC transporter family were reported (Kanno et al. 2012). The *AIT1–4* genes were originally identified using a modified yeast two-hybrid system screen of *Arabidopsis* cDNAs to identify proteins capable of inducing interactions between the ABA receptor PYR/PYL/RCAR and PP2C protein phosphatase under low ABA concentrations. Transport assays in yeast and insect cells expressing candidate genes demonstrated that AIT1, which had been characterised as NRT1.2 and unified as AtNPF4.6 (Léran et al. 2013), mediates cellular ABA uptake. *AIT1* promoter activity was detected in imbibed seeds and vascular tissues of cotyledons, true leaves, hypocotyls, roots and inflorescence stems using a promoter–reporter system. Fluorescent protein-fused AIT1 proteins were detected predominantly at the plasma membrane.

Less sensitivity to exogenously applied ABA during seed germination and/or initial growth was observed in *ait1* mutants compared to wild-type controls, whereas overexpression of AIT1 resulted in ABA hypersensitivity during the same growth stages. In mature plants, the inflorescence stems of *ait1* mutants had

a lower surface temperature than wild-type controls, implying an open-stomata phenotype. These data suggest that the function of AtNPF4.6/NRT1.2/AIT1 as an ABA importer at the site of ABA biosynthesis is important for the regulation of stomatal aperture in shoots.

6 A Current Model

The designation of the three membrane factors, AtABCG25, AtABCG40 and AIT1, as ABA transporters is supported by several lines of evidence (summarized in Table 1). All three proteins were localised to plasma membranes, implying involvement in the transfer of molecules between the inside and outside of cells. All of the gene-disruption mutants showed aberrant sensitivity to ABA accompanied by biochemical evidence of ABA transport activity. Experiments on transported ABA stereospecificity using ABA stereoisomers showed that the transporters had greater substrate affinity for (+)-ABA than for (–)-ABA. The Km saturation kinetics of ATP-dependent ABA transport were not so different for the three proteins (260 nM, 1 μM and 5 μM for AtABCG25, AtABCG40 and AIT1, respectively), although different assay systems were used to calculate the activities (Kang et al. 2010; Kuromori et al. 2010; Kanno et al. 2013). These findings strongly suggest that these three factors mediate active control of ABA transport across cellular membranes.

The AtABCG25 promoter was active in vascular tissue and the AtABCG40 promoter was active in guard cells, which is consistent with a simple model in which vascular tissues are the primary site of ABA synthesis and transport of ABA to guard cells is mediated by specific transporters (Fig. 1; Kuromori and Shinozaki 2010). AtABCG22 may also function in guard cells during osmotic stress responses. This model is consistent with the established cytosolic localisation of receptors that serve as the initial trigger for ABA signalling (Ma et al. 2009; Park et al. 2009). This highlights the potential importance of an ABA transporter that could deliver ABA in a regulated fashion to initiate rapid and controlled responses to various stress conditions (Kang et al. 2010; Kuromori et al. 2010). The ABA importer, AIT1, is expressed in vascular tissues with an expression pattern similar to that of AtABCG25. Accordingly, AIT1 functions to maintain the ABA pool size at the site of biosynthesis, which might be required for the efficient translocation of ABA to guard cells (Fig. 1; Kanno et al. 2012).

However, all of the physiological processes mediated by ABA cannot be fully accounted for by just these three transporters. For example, the phenotypes of the mutants defective in any of the three transporters differed from the phenotypes typical of ABA-deficient mutants, suggesting the possible existence of redundant transporters or a passive transport mechanism mediated by a pH gradient (Sreenivasulu et al. 2012). Alternatively, some physiological responses may not require active ABA transport (Kanno et al. 2012).

Table 1 Comparison of characteristics of the three ABA transporters reported to date

Gene name	AtABCG25	AtABCG40	AtNPF4.6/ NRT1.2/AIT1
Gene family	ABCG half-size type	ABCG full-size type	NRT1/PTR
Gene identification	Mutant screen	Mutant screen	Modified Y2H screen
Tissue expression	Vascular tissues	Broad, highest in guard cells	Vascular tissues
Subcellular localisation	Plasma membrane	Plasma membrane	Plasma membrane
Transport assay	Vesicles from insect cells	Yeast and BY2 cells	Yeast and insect cells
KO phenotype of germinative growth	ABA hypersensitive	ABA insensitive	ABA insensitive
OE phenotype of germinative growth	ABA insensitive	(N.D.)	ABA hypersensitive
Suggested function	Exporter	Importer	Importer

Extracted from Kang et al. (2010), Kuromori et al. (2010), Kanno et al. (2012)
KO knockout mutants, *OE* overexpressed plants, *Y2H* yeast two-hybrid system

Direct orthologues of these ABA transporters are not found in the genome of the moss *Physcomitrella patens* (Sakata et al. 2014), indicating that the *Arabidopsis* ABA transporters described here might have evolved after the emergence of bryophytes. Bryophytes have an ABA responsive system, but they are non-vascular plants. Other types of ABA transporters probably evolved in non-vascular plants.

The current model is based on ABA transport from the site of biosynthesis to the site of action. However, one could ask whether guard cells, as well as vascular cells, are capable of synthesising ABA. Indeed, ABA was shown in some reports to be synthesised in guard cells. For example, reporter gene analysis using the AAO3 and AtNCED3 promoters demonstrated that they are active in guard cells (Tan et al. 2003; Koiwai et al. 2004). More recently, guard cell-autonomous ABA synthesis was reported to be both required and sufficient for stomatal closure in response to low humidity (Bauer et al. 2013). Guard cell-autonomous ABA synthesis allows the plant to respond rapidly to changing environmental conditions to maintain water status homeostasis. In addition, ABA biosynthesis genes and ABA transporter genes tend to show significant expression in roots as well as shoots (Boursiac et al. 2013). A detailed investigation using spatio-temporal analyses of the initial induction of ABA signalling between the site of biosynthesis and the site of action is required.

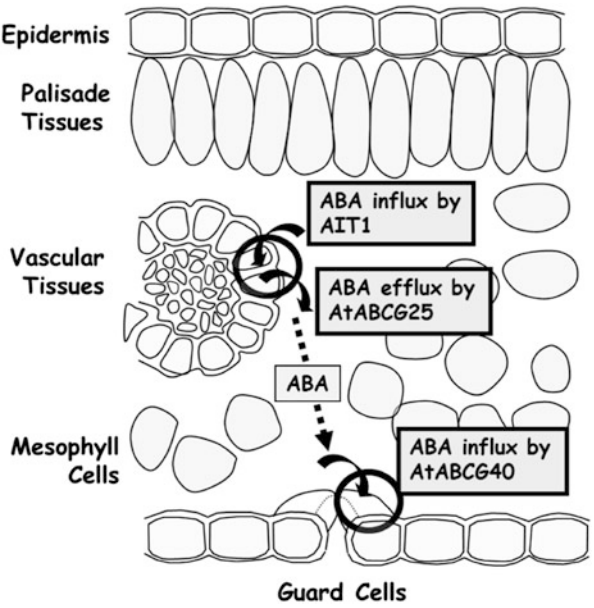

Fig. 1 A current model of ABA transport from vascular tissues to guard cells mediated by specific transporters. A schematic view of a cross section of a plant leaf is shown. AtABCG25 and AIT1 are expressed in vascular tissue and are responsible for ABA efflux and influx, respectively, while AtABCG40 is expressed in guard cells and is responsible for ABA influx. (Modified from Umezawa et al. 2010)

References

Bauer H, Ache P, Lautner S, Fromm J, Hartung W, Al-Rasheid KA, Sonnewald S, Sonnewald U, Kneitz S, Lachmann N, Mendel RR, Bittner F, Hetherington AM, Hedrich R (2013) The stomatal response to reduced relative humidity requires guard cell-autonomous ABA synthesis. Curr Biol 23:53–57

Behnam B, Iuchi S, Fujita M, Fujita Y, Takasaki H, Osakabe Y, Yamaguchi-Shinozaki K, Kobayashi M, Shinozaki K (2013) Characterization of the promoter region of an *Arabidopsis* gene for 9-cis-epoxycarotenoid dioxygenase involved in dehydration-inducible transcription. DNA Res 20:315–324

Benjamins R, Scheres B (2008) Auxin: the looping star in plant development. Annu Rev Plant Biol 59:443–465

Boursiac Y, Leran S, Corratge-Faillie C, Gojon A, Krouk G, Lacombe B (2013) ABA transport and transporters. Trends Plant Sci 18:325–333

Cheng WH, Endo A, Zhou L, Penney J, Chen HC, Arroyo A, Leon P, Nambara E, Asami T, Seo M, Koshiba T, Sheen J (2002) A unique short-chain dehydrogenase/reductase in *Arabidopsis* glucose signaling and abscisic acid biosynthesis and functions. Plant Cell 14:2723–2743

Christmann A, Weiler EW, Steudle E, Grill E (2007) A hydraulic signal in root-to-shoot signalling of water shortage. Plant J 52:167–174

Cutler SR, Rodriguez PL, Finkelstein RR, Abrams SR (2010) Abscisic acid: emergence of a core signaling network. Annu Rev Plant Biol 61:651–679

Endo A, Sawada Y, Takahashi H, Okamoto M, Ikegami K, Koiwai H, Seo M, Toyomasu T, Mitsuhashi W, Shinozaki K, Nakazono M, Kamiya Y, Koshiba T, Nambara E (2008) Drought induction of *Arabidopsis* 9-cis-epoxycarotenoid dioxygenase occurs in vascular parenchyma cells. Plant Physiol 147:1984–1993

Friml J, Palme K (2002) Polar auxin transport—old questions and new concepts? Plant Mol Biol 49:273–284

Graf GA, Yu L, Li WP, Gerard R, Tuma PL, Cohen JC, Hobbs HH (2003) ABCG5 and ABCG8 are obligate heterodimers for protein trafficking and biliary cholesterol excretion. J Biol Chem 278:48275–48282

Hartung W, Wilkinson S, Davies W (1998) Factors that regulate abscisic acid concentrations at the primary site of action at the guard cell. J Exp Bot 49:361–367

Hubbard KE, Nishimura N, Hitomi K, Getzoff ED, Schroeder JI (2010) Early abscisic acid signal transduction mechanisms: newly discovered components and newly emerging questions. Genes Dev 24:1695–1708

Kang J, Hwang JU, Lee M, Kim YY, Assmann SM, Martinoia E, Lee Y (2010) PDR-type ABC transporter mediates cellular uptake of the phytohormone abscisic acid. Proc Natl Acad Sci U S A 107:2355–2360

Kanno Y, Hanada A, Chiba Y, Ichikawa T, Nakazawa M, Matsui M, Koshiba T, Kamiya Y, Seo M (2012) Identification of an abscisic acid transporter by functional screening using the receptor complex as a sensor. Proc Natl Acad Sci U S A 109:9653–9658

Kanno Y, Kamiya Y, Seo M (2013) Nitrate does not compete with abscisic acid as a substrate of AtNPF4.6/NRT1.2/AIT1 in *Arabidopsis*. Plant Signal Behav 8:e26624

Kim TH, Böhmer M, Hu H, Nishimura N, Schroeder JI (2010) Guard cell signal transduction network: advances in understanding abscisic acid, CO_2, and Ca^{2+} signaling. Annu Rev Plant Biol 61:561–591

Klingler JP, Batelli G, Zhu J (2010) ABA receptors: the START of a new paradigm in phytohormone signalling. J Exp Bot 61:3199–3210

Koiwai H, Nakaminami K, Seo M, Mitsuhashi W, Toyomasu T, Koshiba T (2004) Tissue-specific localization of an abscisic acid biosynthetic enzyme, AAO3, in *Arabidopsis*. Plant Physiol 134:1697–1707

Kuromori T, Shinozaki K (2010) ABA transport factors found in *Arabidopsis* ABC transporters. Plant Signal Behav 5:1124–1126

Kuromori T, Miyaji T, Yabuuchi H, Shimizu H, Sugimoto E, Kamiya A, Moriyama Y, Shinozaki K (2010) ABC transporter AtABCG25 is involved in abscisic acid transport and responses. Proc Natl Acad Sci U S A 107:2361–2366

Kuromori T, Sugimoto E, Shinozaki K (2011) *Arabidopsis* mutants of AtABCG22, an ABC transporter gene, increase water transpiration and drought susceptibility. Plant J 67:885–894

Léran S, Varala K, Boyer JC, Chiurazzi M, Crawford N, Daniel-Vedele F, David L, Dickstein R, Fernandez E, Forde B, Gassmann W, Geiger D, Gojon A, Gong JM, Halkier BA, Harris JM, Hedrich R, Limami AM, Rentsch D, Seo M, Tsay YF, Zhang M, Coruzzi G, Lacombe B (2014) A unified nomenclature of NITRATE TRANSPORTER 1/PEPTIDE TRANSPORTER family members in plants. Trends Plant Sci. 19:5–9 doi:10.1016/j.tplants.2013.08.008

Leung J, Giraudat J (1998) Abscisic acid signal transduction. Annu Rev Plant Physiol Plant Mol Biol 49:199–222

Ma Y, Szostkiewicz I, Korte A, Moes D, Yang Y, Christmann A, Grill E (2009) Regulators of PP2C phosphatase activity function as abscisic acid sensors. Science 324:1064–1068

Okamoto M, Tanaka Y, Abrams SR, Kamiya Y, Seki M, Nambara E (2009) High humidity induces ABA 8′-hydroxylase in stomata and vasculature to regulate local and systemic ABA responses in *Arabidopsis*. Plant Physiol 149:825–834

Park SY, Fung P, Nishimura N, Jensen DR, Fujii H, Zhao Y, Lumba S, Santiago J, Rodrigues A, Chow TF, Alfred SE, Bonetta D, Finkelstein R, Provart NJ, Desveaux D, Rodriguez PL, McCourt P, Zhu JK, Schroeder JI, Volkman BF, Cutler SR (2009) Abscisic acid inhibits

type 2C protein phosphatases via the PYR/PYL family of START proteins. Science 324:1068–1071
Petrásek J, Friml J (2009) Auxin transport routes in plant development. Development 136:2675–2688
Raghavendra AS, Gonugunta VK, Christmann A, Grill E (2010) ABA perception and signalling. Trends Plant Sci 15:395–401
Sakata Y, Komatsu K, Takezawa D (2014) ABA as a universal plant hormone. Progress Bot 75:57–96
Samuels L, Kunst L, Jetter R (2008) Sealing plant surfaces: cuticular wax formation by epidermal cells. Annu Rev Plant Biol 59:683–707
Sauter A, Davies WJ, Hartung W (2001) The long-distance abscisic acid signal in the droughted plant: the fate of the hormone on its way from root to shoot. J Exp Bot 52:1991–1997
Seo M, Koshiba T (2011) Transport of ABA from the site of biosynthesis to the site of action. J Plant Res 124:501–507
Sreenivasulu N, Harshavardhan VT, Govind G, Seiler C, Kohli A (2012) Contrapuntal role of ABA: does it mediate stress tolerance or plant growth retardation under long-term drought stress? Gene 506:265–273
Taiz L, Zeiger E (2006) Abscisic acid: a seed maturation and stress signal. Plant Physiology (4th edn) Chap. 23
Tan BC, Joseph LM, Deng WT, Liu LJ, Li QB, Cline K, McCarty DR (2003) Molecular characterization of the *Arabidopsis* 9-cis epoxycarotenoid dioxygenase gene family. Plant J 35:44–56
Tanaka H, Dhonukshe P, Brewer PB, Friml J (2006) Spatiotemporal asymmetric auxin distribution: a means to coordinate plant development. Cell Mol Life Sci 63:2738–2754
Umezawa T, Nakashima K, Miyakawa T, Kuromori T, Tanokura M, Shinozaki K, Yamaguchi-Shinozaki K (2010) Molecular basis of the core regulatory network in ABA responses: sensing, signaling and transport. Plant Cell Physiol 51:1821–1839
Vanneste S, Friml J (2009) Auxin: a trigger for change in plant development. Cell 136:1005–1016
Verrier PJ, Bird D, Burla B, Dassa E, Forestier C, Geisler M, Klein M, Kolukisaoglu U, Lee Y, Martinoia E, Murphy A, Rea PA, Samuels L, Schulz B, Spalding EJ, Yazaki K, Theodoulou FL (2008) Plant ABC proteins—a unified nomenclature and updated inventory. Trends Plant Sci 13:151–159

Exine Export in Pollen

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Abstract Pollen as a sperm cell carrier is mainly protected by outer pollen wall (called exine) from physical and biological stresses. The major composition of exine is the highly resistant biopolymer sporopollenin, which mainly consists of hydrophobic lipids, phenylpropanoids, and aromatic compounds. The biosynthesis of these constituents has been shown to be catalyzed by enzymes preferentially expressed in the sporophytic tapetal layer, a nutritive tissue supporting pollen development. How the synthesized sporopollenin precursors are exported from tapetal cells onto the surface of microspore for pollen exine formation remains largely unknown. Here, we review the structure of tapetal cella and pollen exine in the model monocot rice (*Oryza sativa*) and the model dicot *Arabidopsis thaliana*. In addition, we highlight the update understanding on the role of ATP-binding cassette (ABC), lipid transfer protein (LTP), and multidrug and toxic efflux (MATE) transporters in trafficking of sporopollenin precursors across tapetal cells for exine development in rice and *Arabidopsis*. We also discuss the future research focus on the transport of sporopollenin precursors for exine synthesis.

1 Introduction

Successful reproduction in flowering plants relies on the interaction between male and female gametophytes. Unlike the female gametes embedded by other plant tissues, the mature pollen grain as plant male gamete released from the anther is

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required to be distributed and adhered onto the female tissue by insects, wind, and other vectors. To survive on the dispersal pathway, pollen grains develop the pollen wall that comprises three layers: pollen coat, the outer pollen wall (also called exine), and inner intine layer (Zinkl et al. 1999). Exine has extremely physical and chemical stability resistant to biotic and abiotic stresses such as high temperature, desiccation, ultraviolet (UV) irradiation, and mechanical damage, etc. Additionally, during fertilization the pollen wall plays a critical role in pollen–stigma communication and pollen rehydration and acts as a possible reproductive barrier to be recognized as self and non-self pollen grains by female tissue in plants (Zinkl et al. 1999). In plant kingdom, pollen grains exhibit a great biodiversity in shape and surface patterning. The model dicot plant *Arabidopsis thaliana* has ellipsoidal pollen grains covered by reticulate surface distributed by insects, while the model monocot plant rice (*Oryza sativa*) has globular pollen grains with smooth appearance transmitted by wind (Fig. 1a, b). Due to its biological importance, the mechanism underlying the biosynthesis of pollen wall, particularly exine, is an interesting biological question to be addressed for plant biologists.

2 Structure and Development of Exine

2.1 Exine Structure

Although the morphology of pollen grains varies among different species, the pollen wall structure is mainly consisted of layers such as intine, exine, and pollen coat, from the inside to the outside. Exine generally contains nexine, bacula, and tectum (Fig. 1c–f) (Zhang et al. 2011). *Arabidopsis* exine has a thin nexine layer, a semi-open tectum layer, and a longer baculum (Fig. 1c, e) compared with rice in which its pollen exine has a thick tectum and nexine and a high density of bacula (Fig. 1d, f).

2.2 Formation of Exine

Developmentally most plant species share similar biological processes in exine biosynthesis even though different plants exhibit a diversity of pollen grain morphology (Zhang et al. 2011; Ariizumi and Toriyama 2011; Li and Zhang 2010; Blackmore et al. 2007; Wilson and Zhang 2009). The formation of pollen wall starts before meiosis as evidenced from rice and *Arabidopsis* (Zhang et al. 2011; Ariizumi and Toriyama 2011; Li and Zhang 2010; Blackmore et al. 2007; Wilson and Zhang 2009). According to the classification of rice anther development stage and pollen exine formation, we propose a schematic model illustrating exine developmental events (Fig. 2). At stage 6 of anther development before the meiosis of pollen

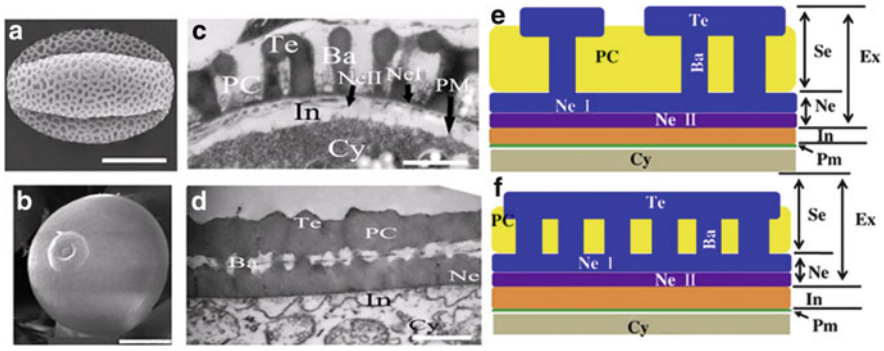

Fig. 1 Pollen wall morphology of rice and *Arabidopsis* at mature stage. (**a** and **b**) Pollen grains of *Arabidopsis* (**a**) and rice (**b**) by SEM, bar = 5 mm. (**c** and **d**) Transverse section of pollen wall of *Arabidopsis* (**c**) and rice (**d**) by transmission electron microscope, bar = 1 mm. (**e** and **f**) Proposed model of pollen wall structure of *Arabidopsis* (**e**) and rice (**f**). *In* intine, *Ne* nexine, *Ex* exine, *Se* sexine, *Te* tectum, *Ba* bacula, *PC* pollen coat, *PM* plasma membrane, *M* microspore, *Cy* cytoplasm. Figure taken from Zhang et al. (2011)

mother cells (PMCs), the anther initiates the synthesis of callose (a b-1,3-linked glucan) deposited onto PMCs (Fig. 2-A1 and E1). During the meiosis (stage 7), PMC produces the tetrad and each contains four newly formed microspores enclosed by callose, and each microspore forms the primexine with the low electron density surrounding its outer surface (Fig. 2-A2 and E2). Primexine is a microfibrillar matrix mainly consisting of cellulose synthesized by the microspores and serves as an elaborate template patterning for the deposition of sporopollenin precursors and the following polymerization (Blackmore et al. 2007). At stage 8 during the tetrad formation, the probaculum is formed and deposited onto the primexine (Fig. 2-A3 and E3). After the release from the tetrad by degeneration of callose wall at stage 9, microspores are enclosed by a very thin tectum (sexine) on the primexine as the deposition of sporopollenin precursors. Meanwhile, the formation of nexine (the foot layer) is seen on the surface of microspores. Subsequently sporopollenin precursors continue the deposition onto the tectum (Fig. 2-A4 and E4). As the development of microspore, pollen exine becomes thick and consolidates as the accumulation of sporopollenin precursors (Fig. 2-A5 and E5). At stage 10, the microspore becomes vacuolated and the pollen exine displays a two-layer structure with low-electron density intervals or channels across the pollen wall (Fig. 2-A6 and E6). Subsequently, during the mitosis each microspore generates two sperms and one vegetative nucleus, and the microspore starts the synthesis of intine below the exine. Till stage 12 when mature pollen grains form, the pollen exine ontology is almost established by the accumulation of sporopollenin compositions (Fig. 2-A7 and E7). Finally, before the release of pollen grains from anther locule, tryphine is deposited as a compound of pollen coat at stage13 (Fig. 2-A8 and E8).

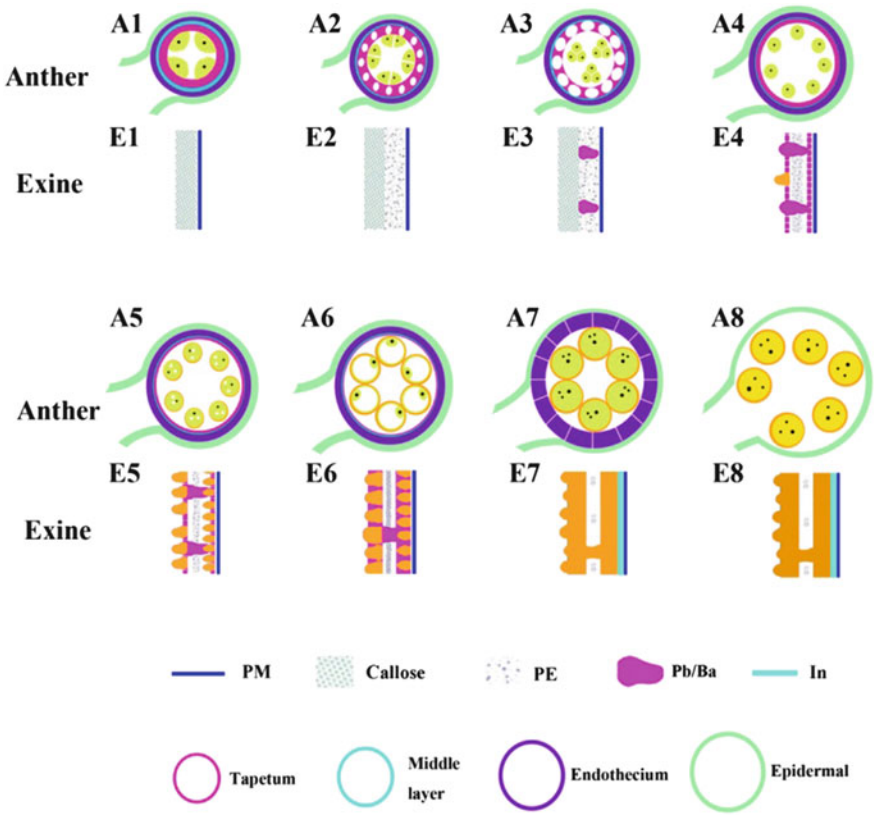

Fig. 2 Schematic model of pollen development and pollen wall formation within rice anther. A1–A8: Cytological profile and diagrams of rice anther development and pollen formation from meiosis stage to mature pollen stage. The definition of anther developmental stage refers to (Zhang et al. 2011) E1–E8. The model of rice pollen wall development at the corresponding stage of the A1–A8 for anther development. Figure modified from Li and Zhang (2010)

3 Tapetum: The Supportive Tissue for Exine Formation

3.1 Secretory Structures of Tapetal Cells in Plants

According to the morphogenesis and cellular features of anther development from rice and *Arabidopsis*, pollen exine development seems to be closely related to anther sporophytic cell degeneration (Fig. 2) (Ma 2005; Zhang and Wilson 2009; Li et al. 2010; Wang et al. 2003). During early stage, the anther primordium undergoes cell division and cell differentiation generating four-layer anther wall, i.e., the epidermis, endothecium, middle layer, and tapetum from the outside to the inside, as well as sporophytic PMCs (Fig. 2-A1). After meiosis, tapetal cells undergo programmed cell death (PCD)-promoted degeneration (Fig. 2-A2),

which is assumed to provide nutritive support for pollen maturation including exine formation (Fig. 2-A3 to A8).

Even though both rice and *Arabidopsis* develop secretory tapeta (Huysmans et al. 1998; Furness and Rudall 2001), the inner side of the tapetal cells in rice and other cereals exhibit characteristic orbicules/Ubisch bodies facing to male reproductive cells (Fig. 3a, b), which are assumed to transfer tapetum-synthesized sporopollenin precursors across the hydrophilic cell wall to the anther locule (Ting et al. 1998; Hsieh and Huang 2007). However, orbicules were not seen in *Brassicaceae* species, such as Arabidopsis, which has unique secretory tapetal cells containing specialized lipidic organelles elaioplasts and tapetosomes (Fig. 3c, d) (Hsieh and Huang 2007). The ER-derived spherical tapetosomes consist of flavonoids, alkanes, and oleosins and are responsible for the transport of pollen wall constituents (Hsieh and Huang 2007). Currently, the detailed role of these specialized organelles in exporting sporopollenin precursors for exine development is unclear.

3.2 Tapetal Programmed Cell Death Is Associated With Exine Formation

Exine synthesis and patterning rely on tapetal PCD, which is controlled by the functions of regulators such as basic helix–loop–helix (bHLH) transcription factors, MYB transcription factor, and PHD-finger protein (Yang et al. 2007; Aya et al. 2009; Li et al. 2006; Xu et al. 2010; Sorensen et al. 2003). Loss of function of these regulators frequently causes defective tapetal PCD and abnormal exine formation (Yang et al. 2007; Aya et al. 2009; Li et al. 2006; Xu et al. 2010; Sorensen et al. 2003). These findings suggest the possible close link between tapetal PCD and nutritive support for exine biosynthesis. However it is not clear whether these two processes function in the same pathway or in parallel.

4 Biosynthesis of Exine

The biopolymer sporopollenin has been regarded as a major component of exine of plant spores and pollen grains (Scott 1994), but the chemical nature for sporopollenin is not exactly known due to no available approaches capable of isolating sporopollenin monomers. Even though sporopollenin is highly insoluble and hardly to be degraded and analyzed, increasing evidence shows that the sporopollenin is an aliphatic polymer comprising of long-chain fatty acids, phenylpropanoids, and oxygenated aromatic rings (Scott 1994; Piffanelli et al. 1998). In addition, the diploid sporophytic tapetal cells act as a major tissue synthesizing sporopollenin precursors for exine synthesis (Piffanelli et al. 1998).

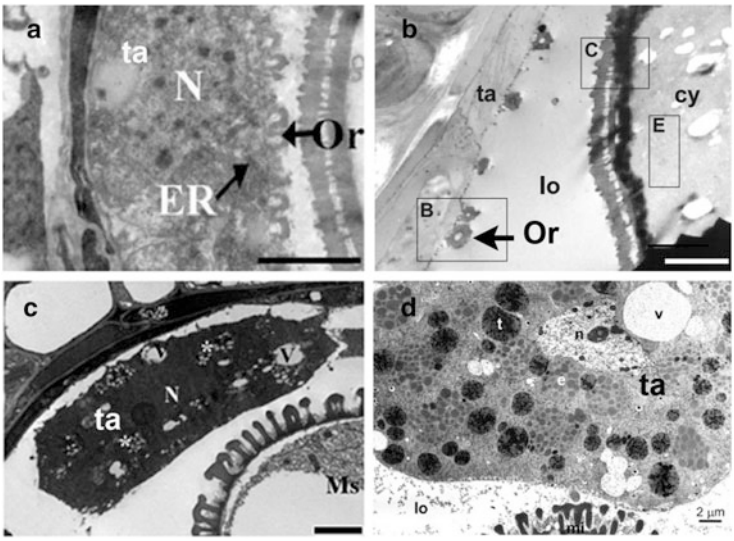

Fig. 3 Structural difference in tapetal cells between cereals and *Brassicaceae*. (**a** and **b**) Orbicule distributed on the inner surface of tapetum adjacent to the anther locule in rice (**a**) and wheat (**b**). (**a**) is from (Li and Zhang 2010), Bar = 2 μm; (**b**) is from (Wang et al. 2003), Bar = 3 μm. (**c** and **d**) Tapetosomes (t) and elaioplasts (e) within the tapetum cell of *Arabidopsis* (**c**) and *Brassica napus* (**d**). (**c**) is from (Yang et al. 2007), Bar = 2 μm; (**d**) is from (Hsieh and Huang 2007), Bar = 2 μm. *Or* orbicule, *ta* tapetum, *N* nucleus, *V* vacuolar, *Ms* microspore, *asterisks* plastid inclusions

4.1 Synthesis of Lipidic Molecules

Biochemical and genetic investigations revealed that fatty acids and their derivatives catalyzed by a series of enzymes are key compositions of sporopollenin (Fig. 4) (Li et al. 2010; Aarts et al. 1997; Morant et al. 2007; Dobritsa et al. 2009, 2010; Jung et al. 2006; Souza et al. 2009; Chen et al. 2011; Shi et al. 2011; Kim et al. 2010; Tang et al. 2009; Grienenberger et al. 2010). In heterotrophic eukaryotes, the synthesis of fatty acids usually occurs in the cytosol, whereas plants produce long-chain fatty acids up to C18 mainly in plastids, such as the chloroplast (Ohlrogge et al. 1979; Li-Beisson et al. 2010), and some fatty acids are modified in the endoplasmic reticulum (ER) (Kunst and Samuels 2003). Plastids have been proved to be crucial for successful anther development in angiosperms, including synthesis of fatty acids and alcohols (Chen et al. 2011; Shi et al. 2011) and storage lipids.

Rice Defective Pollen Wall (DPW) and *Arabidopsis* counterpart male sterility 2 (MS2) are the plastid-localized reductases expressed in tapetal cells which have biochemical activities of converting long-chain fatty acids of 16 carbon and 18 carbon into fatty alcohols (Chen et al. 2011; Shi et al. 2011). Rice Wax-Deficient Anther1 (WDA1) and its homolog ECERIFERUM1 (CER1) in *Arabidopsis* were shown to participate in the metabolism of very-long-chain fatty acids for the

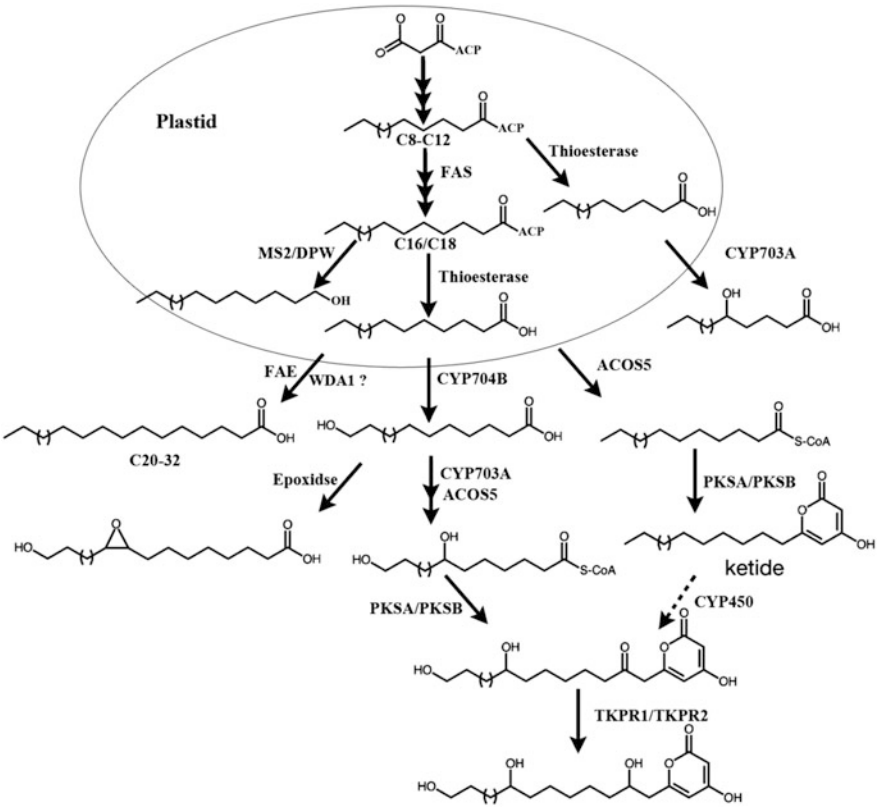

Fig. 4 The biochemical pathway of lipidic and aromatic metabolism for sporopollenin biosynthesis in plants. In plastids, de novo synthesized fatty acids are esterified to acyl carrier protein (ACP), and the long-chain acyl-ACPs are cleaved, transferred from the plastid to ER, and connected to CoA for elongation (Li-Beisson et al. 2010). C16:0-ACP is reduced to hexadecanol by rice DPW (Shi et al. 2011) and Arabidopsis MS2 (Chen et al. 2011) in plastids, and hexadecanol can freely diffuse or be transported into the cytoplasm (Spector and Soboroff 1972). Hexadecanol can be further converted to fatty acid and oxygenated by ER-localized P450 enzymes. The hydroxylated fatty acids can then be converted to acyl-CoAs by ACOS (Souza et al. 2009), and acyl-CoAs can be converted to polyketides by TKPR1/2 (Grienenberger et al. 2010), PKSA/LAP6, and PKSB/LAP5 (Kim et al. 2010). Figure modified from (Shi et al. 2011; Kim et al. 2010; Grienenberger et al. 2010)

establishment of the anther cuticle and pollen exine (Jung et al. 2006). Members of cytochrome P450s such as CYP703A and CYP704B subfamilies have key roles in hydroxylating fatty acids in ER for exine formation (Li et al. 2010; Morant et al. 2007; Dobritsa et al. 2009). CYP703As catalyze the in-chain hydroxylation of lauric acids (7OH-C12) and CYP704Bs produce ω-hydroxylated fatty acids with 16 and 18 carbons (ω-OH-C16 and ω-OH-C18) as building blocks for sporopollenin assembly (Morant et al. 2007; Dobritsa et al. 2009). The hydroxylated fatty acids by CYP703A and CYP704B can be further catalyzed by cytoplasm-localized fatty

acyl-CoA synthetase (ACOS) to generate CoA esters for sporopollenin synthesis (Fig. 4) (Souza et al. 2009).

4.2 Phenylpropanoids and Aromatic Metabolism

Beside fatty acids, aromatic polyketides such as tri- and tetraketide alpha-pyrones were identified as sporopollenin constituents catalyzed by *Arabidopsis* ER-localized LAP6 (LESS ADHESIVE POLLEN)/PKSA (POLYKETIDE SYNTHASE A) and LAP5/PKSB using the substrates produced by ACOS5 (Fig. 3) (Dobritsa et al. 2010; Kim et al. 2010). Next, tri- and tetraketide alpha-pyrones as the substrate can be further derived into hydroxylated alpha-pyrone by the reductase TETRAKETIDE α-PYRONE REDUCTASE1 (TKPR1) and TKPR2 for the sporopollenin monomer biosynthesis (Grienenberger et al. 2010). These findings reveal the phenylpropanoids and aromatic compounds as key sporopollenin components for exine formation (Fig. 4) (Ahlers et al. 1999).

5 Carriers for Exine Export

How tapetum-derived chemicals are exported into the locule for the assembly of sporopollenin is a mysterious question for biologists. Up to date, ATP cassette (ABC) and lipid transfer protein (LTP) transporters are molecules proved to be responsible for cargo transport of exine constituents.

5.1 ABC Transporters

Plant ABC transporters have been shown to be involved in a wide range of cellular activity, such as hormone homeostasis, detoxification, antibiotics resistance, secondary metabolism, etc. (Kang et al. 2011). Among these ABC transporters, some G subfamily members such as *Arabidopsis ABCG26* (*WBC27*) and rice ABCG15 (called *Post-meiotic Deficient Anther 1, PDA1*) expressed in tapetal cells are assumed to be responsible for transport of sporopollenin precursors from tapetal cells into microspore surface for exine development. Knockdown of *ABCG26* (*WBC27*) leads to the aborted exine formation, leaving abnormal aggregated materials on the microspore surface and collapsed microspores (Xu et al. 2010; Dou et al. 2011; Quilichini et al. 2010; Choi et al. 2011; Kuromori et al. 2010). These findings suggest that the ABC transporter ABCG26 (WBC27) plays a key role in exine formation, likely secreting the sporopollenin precursors from the tapetum to developing microspores. Moreover, the bHLH transcription factor ABORTED MICROSPORES (AMS) directly regulates the expression of *ABCG26* via binding

to *ABCG26* promoter and coordinates the synthesis of aliphatic molecules in the tapetum (Xu et al. 2010).

Recently, rice *ABCG15*/*PDA1*, a close homolog of *ABCG26*, has been shown to be required for exine development (Qin et al. 2013; Zhu et al. 2013). Mutation of the tapetum-expressed *PDA1* causes the abortion of microspore development without exine formation, no orbicules on tapetal cells in the mutants (Qin et al. 2013; Zhu et al. 2013). The amount of anther cuticular wax and cutin is reduced in the *pda1* mutant, suggesting that PDA1-mediated transport of sporepollenin precursors feedback affects the synthesis of lipidic components. Consistently, the expression of two pollen exine biosynthetic genes *CYP704B2* and *CYP703A3* decreased in *pda1* anthers. These findings suggest that the tapetum-expressed ABC transporters play a conserved role in secreting lipidic sporopollenin precursors from the tapetum to developing microspores in both rice and *Arabidopsis*. However, the biochemical evidence on how ABC transporters translocate sporopollenin precursors and what are their exact substrates remain to be investigated. Future biochemical function assay of ABCG26/ABCG15 may help us in understanding the role of ABC transporter in plant exine development.

5.2 Lipid Transfer Proteins

Besides the possible role of transmembrane ABCG transporters in actively delivering the lipid molecules across the membrane, other lipid transporters such as low molecular weight LTPs have been shown to be involved in lipid trafficking (Huang et al. 2013; Zhang et al. 2010; Yeats and Rose 2008; Kader 1996). LTPs were termed as they have the capacity to transfer phospholipids and fatty acids between membranes in vitro (Kader 1996). Expression analysis showed that some tapetal PCD regulators affect the expression of LTPs (Aya et al. 2009; Li et al. 2006, 2011; Xu et al. 2010; Jung et al. 2005), implying that LTPs regulate pollen development.

One rice LTP, OsC6, was shown to function as lipidic transporter from tapetal cells to anther wall layers and microspore surface (Zhang et al. 2010). OsC6 has the typical eight-Cys motif, and its transcription is highly detectable in tapetal cell. However, the localization of OsC6 protein is seen in tapetal cells, anther locule, anther epidermis, as well as the extracellular space between the tapetum and the middle layer, indicating that the tapetum expressed OsC6 can be secreted among anther cells. In addition, the recombinant OsC6 protein has the bind ability of fatty acid molecules (Zhang et al. 2010), suggesting that OsC6 is involved in distribution of lipidic molecules for male reproduction. Furthermore, knockdown of *OsC6* caused aborted exine formation and orbicule development in rice. This work demonstrates the key role of LTPs in secreting lipidic molecule from the tapetum for exine development. Recently, Huang et.al (2013) also demonstrated that the tapetum-expressed type- III LTP plays a role for the lipid molecular trafficking from tapetum into the pollen exine in *Arabidopsis*, confirming the novel vesicular

trafficking mechanism for pollen exine development (Huang et al. 2013). However, the substrates of LTPs remain to be analyzed in the future.

5.3 *Other Putative Transporters*

Evidence shows that the flavonoids and lipid droplet in tapetosomes are likely used as exine and pollen coat constituents in Brassica (Hsieh and Huang 2007). Moreover, the aromatic chemicals synthesized by LAP5/6(PKSA/B) and TKPR1/2 are essential for pollen exine formation. However, it remains largely unknown how these compounds are transported for pollen exine synthesis. During the development of seeds or leaf color, multidrug and toxic efflux (MATE) transporter TT12 (Transparent Testa12) and its putative cofactor glutathione S-transferase (GST) TT19 were identified to be responsible for the transport of phenolic compounds (Sun et al. 2012; Zhao and Dixon 2009; Marinova et al. 2007; Kitamura et al. 2004). Yeast-expressed TT12, a vacuolar flavonoid/H^+-antiporter, exhibits high affinity to catechin-3-*O*-glucoside, a glucosylated flavan-3-ol, and TT12 enhances the vacuolar uptake of anthocyanin in *Arabidopsis* seeds (Marinova et al. 2007). GST protein encoded by TT19 regulates the anthocyanin trafficking into the vacuole in *Arabidopsis* hypocotyls and cotyledons (Kitamura et al. 2004). Whether these transporters are required for the transport of phenolic compounds for exine synthesis remains unknown because of no direct genetic evidence. Hsieh and Huang (Hsieh and Huang 2007) investigated the distribution of flavonoids in tapetal cells in the *tt12* and *tt19* mutant and showed that flavonoids were observed in the cytosol and not in the tapetosomes in the *tt12* and *tt19* tapetal cells and also decreased amounts of flavonoids in the mutant tapetal cells and pollen surface compared to the wild type (Hsieh and Huang 2007), implying that MATE transporter and GST proteins may be involved in the phenolic trafficking from tapetal cells for pollen exine or coat formation.

Based on the above genetic, molecular, and biochemical investigations, we propose a putative model on the possible trafficking pathways involved in exporting sporopollenin precursors from tapetal cells to anther locule for exine synthesis in plants (Fig. 5). The synthetic aliphatic or aromatic constituents catalyzed by the ER-localized enzymes such as CYP450, PKS, and TKPR are secreted by endoplasmic reticulum (ER)-derived vesicles from the synthetic site, then some compounds are delivered into Golgi for further modification or the vesicles are aggregated as suborganelles such as tapetosomes or orbicules. The exocytosis of sporopollen precursors in lipidic suborganelles is affected through the fusion with the plasma membrane of tapetum cells. Additionally, other transporters, such as ABC transporters and LTPs, may be involved in the precursor export.

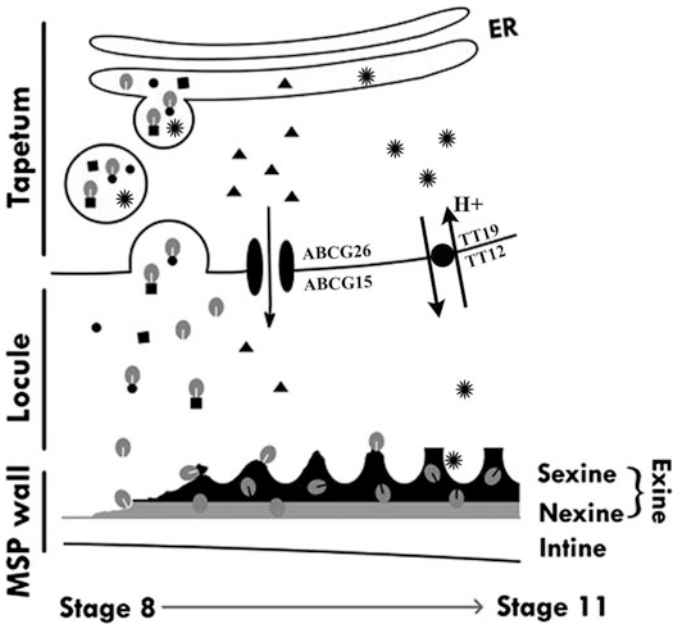

Fig. 5 Proposed model for exporting sporopollenin constituents from tapetal cells onto the microspore surface for exine formation. Fatty acids, alcohols, polyketides, and other derived monomers may be translocated from the tapetal cells by ATP-binding cassette transporters, such as WBC27/ABCG26 and ABCG15 (Xu et al. 2010; Dou et al. 2011; Quilichini et al. 2010; Choi et al. 2011; Qin et al. 2013; Zhu et al. 2013), and/or lipid transport proteins, such as OsC6 (Zhang et al. 2010), and type III lipid transport protein (Huang et al. 2013) into the locule as sporopollenin precursors for the assembly of pollen exine in plants. MATE transporters might be also involved in the precursor transportation. Lipid transport proteins ; Aliphatic compound, ▲ ■ ●; Aromatic compound, ✸. Modified from Huang et al. (2013)

6 Perspective

Lipidic and phenolic molecules secreted from tapetum cell are considered to be the key compounds determining the framework for pollen exine. Even though this process is essential for reproduction success in flowering plants, many questions remain to be addressed. For instance, what is the link between tapetal PCD and the supply of sporopollen precursors? Which molecules coordinate the developmental programs between the microspore and tapetal cells? Furthermore, during the microspore development, the microspore always attaches to the inner side of tapetal cells, whether the liquid in the anther locule plays a short-distance transport role for exine formation? Moreover, the substrates of different transport machineries/molecules during exine formation remain to be elucidated. As the advance of the tools for cell biology, biochemistry, more understandings will be revealed on the mechanism underlying the synthesis and transport of sporopollenin precursors during plant exine formation.

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References

Aarts MG, Hodge R, Kalantidis K, Florack D, Wilson ZA, Mulligan BJ, Stiekema WJ, Scott R, Pereira A (1997) The Arabidopsis male sterility 2 protein shares similarity with reductases in elongation/condensation complexes. Plant J 12:615–623

Ahlers F, Thom I, Lambert J, Kuckuk R, Wiermann R (1999) ^{1}H NMR analysis of sporopollenin from *Typha Angustifolia*. Phytochemistry 50:1095–1098

Ariizumi T, Toriyama K (2011) Genetic regulation of sporopollenin synthesis and pollen exine development. Annu Rev Plant Biol 62:437–460

Aya K, Ueguchi-Tanaka M, Kondo M, Hamada K, Yano K, Nishimura M, Matsuoka M (2009) Gibberellin modulates anther development in rice via the transcriptional regulation of GAMYB. Plant Cell 21:1453–1472

Blackmore S, Wortley AH, Skvarla JJ, Rowley JR (2007) Pollen wall development in flowering plants. New Phytologist 174:483–498

Chen W, Yu X-H, Zhang K, Shi J, De Oliveira S, Schreiber L, Shanklin J, Zhang D (2011) *Male Sterile2* encodes a plastid-localized fatty acyl carrier protein reductase required for pollen exine development in Arabidopsis. Plant Physiol 157:842–853

Choi H, Jin JY, Choi S, Hwang JU, Kim YY, Suh MC, Lee Y (2011) An ABCG/WBC-type ABC transporter is essential for transport of sporopollenin precursors for exine formation in developing pollen. Plant J 65:181–193

Dobritsa AA, Shrestha J, Morant M, Pinot F, Matsuno M, Swanson R, Møller BL, Preuss D (2009) CYP704B1 is a long-chain fatty acid ω-hydroxylase essential for sporopollenin synthesis in pollen of Arabidopsis. Plant Physiol 151:574–589

Dobritsa AA, Lei Z, Nishikawa S-I, Urbanczyk-Wochniak E, Huhman DV, Preuss D, Sumner LW (2010) *LAP5* and *LAP6* encode anther-specific proteins with similarity to chalcone synthase essential for pollen exine development in Arabidopsis. Plant Physiology 153:937–955

Dou XY, Yang KZ, Zhang Y, Wang W, Liu XL, Chen LQ, Zhang XQ, Ye D (2011) WBC27, an adenosine tri-phosphate-binding cassette protein, controls pollen wall formation and patterning in Arabidopsis. J Integr Plant Biol 53:74–88

Furness CA, Rudall PJ (2001) The tapetum in basal angiosperms: early diversity. Int J Plant Sci 162:375–392

Grienenberger E, Kim SS, Lallemand B, Geoffroy P, Heintz D, de Azevedo Souza C, Heitz T, Douglas CJ, Legrand M (2010) Analysis of TETRAKETIDE α-PYRONE REDUCTASE function in *Arabidopsis thaliana* reveals a previously unknown, but conserved, biochemical pathway in sporopollenin monomer biosynthesis. Plant Cell 22:4067–4083

Hsieh K, Huang AH (2007) Tapetosomes in Brassica tapetum accumulate endoplasmic reticulum–derived flavonoids and alkanes for delivery to the pollen surface. Plant Cell 19:582–596

Huang MD, Chen TL, Huang AH (2013) Abundant type III lipid transfer proteins in Arabidopsis tapetum are secreted to the locule and become a constituent of the pollen exine. Plant Physiol 163:1218–1229

Huysmans S, El-Ghazaly G, Smets E (1998) Orbicules in angiosperms: morphology, function, distribution, and relation with tapetum types. Bot Rev 64:240–272

Jung K-H, Han M-J, Lee Y-S, Kim Y-W, Hwang I, Kim M-J, Kim Y-K, Nahm BH, An G (2005) Rice *Undeveloped Tapetum1* is a major regulator of early tapetum development. Plant Cell 17:2705–2722

Jung K-H, Han M-J, Lee D-Y, Lee Y-S, Schreiber L, Franke R, Faust A, Yephremov A, Saedler H, Kim Y-W, Hwang I, An G (2006) *Wax-deficient anther1* is involved in cuticle and wax production in rice anther walls and is required for pollen development. Plant Cell 18:3015–3032

Kader J-C (1996) Lipid-transfer proteins in plants. Annu Rev Plant Biol 47:627–654

Kang J, Park J, Choi H, Burla B, Kretzschmar T, Lee Y, Martinoia E (2011) Plant ABC transporters. The Arabidopsis book/American Society of Plant Biologists 9

Kim SS, Grienenberger E, Lallemand B, Colpitts CC, Kim SY, de Azevedo Souza C, Geoffroy P, Heintz D, Krahn D, Kaiser M (2010) *LAP6*/*POLYKETIDE SYNTHASE A* and *LAP5*/*POLYKETIDE SYNTHASE B* encode hydroxyalkyl α-pyrone synthases required for pollen development and sporopollenin biosynthesis in Arabidopsis thaliana. Plant Cell 22:4045–4066

Kitamura S, Shikazono N, Tanaka A (2004) TRANSPARENT TESTA 19 is involved in the accumulation of both anthocyanins and proanthocyanidins in Arabidopsis. The Plant Journal 37:104–114

Kunst L, Samuels A (2003) Biosynthesis and secretion of plant cuticular wax. Progres Lipid Res 42:51–80

Kuromori T, Miyaji T, Yabuuchi H, Shimizu H, Sugimoto E, Kamiya A, Moriyama Y, Shinozaki K (2010) ABC transporter AtABCG25 is involved in abscisic acid transport and responses. Proc Natl Acad Sci 107:2361–2366

Li H, Zhang D (2010) Biosynthesis of anther cuticle and pollen exine in rice. Plant Signal Behav 5:1121–1123

Li N, Zhang D-S, Liu H-S, Yin C-S, Li X-X, Liang W-Q, Yuan Z, Xu B, Chu H-W, Wang J, Wen T-Q, Huang H, Luo D, Ma H, Zhang D-B (2006) The rice *Tapetum Degeneration Retardation* gene is required for tapetum degradation and anther development. Plant Cell 18:2999–3014

Li H, Pinot F, Sauveplane V, Werck-Reichhart D, Diehl P, Schreiber L, Franke R, Zhang P, Chen L, Gao Y (2010) Cytochrome P450 family member CYP704B2 catalyzes the ω-hydroxylation of fatty acids and is required for anther cutin biosynthesis and pollen exine formation in rice. Plant Cell 22:173–190

Li H, Yuan Z, Vizcay-Barrena G, Yang C, Liang W, Zong J, Wilson ZA, Zhang D (2011) *PERSISTENT TAPETAL CELL1* encodes a PHD-finger protein that is required for tapetal cell death and pollen development in rice. Plant Physiol 156:615–630

Li-Beisson Y, Shorrosh B, Beisson F, Andersson MX, Arondel V, Bates PD, Baud S, Bird D, DeBono A, Durrett TP (2010) Acyl-lipid metabolism. The Arabidopsis Book/American Society of Plant Biologists 8

Ma H (2005) Molecular genetic analyses of microsporogenesis and microgametogenesis in flowering plants. Annu Rev Plant Biol 56:393–434

Marinova K, Pourcel L, Weder B, Schwarz M, Barron D, Routaboul J-M, Debeaujon I, Klein M (2007) The Arabidopsis MATE transporter TT12 acts as a vacuolar flavonoid/H+-antiporter active in proanthocyanidin-accumulating cells of the seed coat. Plant Cell 19:2023–2038

Morant M, Jørgensen K, Schaller H, Pinot F, Møller BL, Werck-Reichhart D, Bak S (2007) CYP703 is an ancient cytochrome P450 in land plants catalyzing in-chain hydroxylation of lauric acid to provide building blocks for sporopollenin synthesis in pollen. Plant Cell 19:1473–1487

Ohlrogge JB, Kuhn DN, Stumpf P (1979) Subcellular localization of acyl carrier protein in leaf protoplasts of Spinacia oleracea. Proc Natl Acad Sci 76:1194–1198

Piffanelli P, Ross JH, Murphy D (1998) Biogenesis and function of the lipidic structures of pollen grains. Sex Plant Reprod 11:65–80

Qin P, Tu B, Wang Y, Deng L, Quilichini TD, Li T, Wang H, Ma B, Li S (2013) *ABCG15* encodes an ABC transporter protein, and is essential for post-meiotic anther and pollen exine development in rice. Plant Cell Physiol 54:138–154

Quilichini TD, Friedmann MC, Samuels AL, Douglas CJ (2010) ATP-binding cassette transporter G26 is required for male fertility and pollen exine formation in Arabidopsis. Plant Physiol 154:678–690

Scott R (1994) Pollen exine-the sporopollenin enigma and the physics of pattern. In: Seminar series-society for experimental biology, Vol 55. (Cambridge University Press), pp 49–49

Shi J, Tan H, Yu X-H, Liu Y, Liang W, Ranathunge K, Franke RB, Schreiber L, Wang Y, Kai G, Shanklin J, Ma H, Zhang D-B (2011) *Defective Pollen Wall* is required for anther and microspore development in rice and encodes a fatty acyl carrier protein reductase. Plant Cell 23:2225–2246

Sorensen AM, Kröber S, Unte US, Huijser P, Dekker K, Saedler H (2003) The Arabidopsis *ABORTED MICROSPORES (AMS)* gene encodes a MYC class transcription factor. Plant J 33:413–423

Souza C, Kim SS, Koch S, Kienow L, Schneider K, McKim SM, Haughn GW, Kombrink E, Douglas CJ (2009) A novel fatty Acyl-CoA synthetase is required for pollen development and sporopollenin biosynthesis in Arabidopsis. Plant Cell 21:507–525

Spector AA, Soboroff JM (1972) Studies on the cellular mechanism of free fatty acid uptake using an analog, hexadecanol. J Lipid Res 13:790–796

Sun Y, Li H, Huang J-R (2012) Arabidopsis TT19 functions as a carrier to transport anthocyanin from the cytosol to tonoplasts. Mol Plant 5:387–400

Tang LK, Chu H, Yip WK, Yeung EC, Lo C (2009) An anther-specific *Dihydroflavonol 4-Reductase-Like* gene (DRL1) is essential for male fertility in Arabidopsis. New Phytologist 181:576–587

Ting JT, Wu SS, Ratnayake C, Huang AH (1998) Constituents of the tapetosomes and elaioplasts in Brassica campestristapetum and their degradation and retention during microsporogenesis. Plant J 16:541–551

Wang A, Xia Q, Xie W, Datla R, Selvaraj G (2003) The classical Ubisch bodies carry a sporophytically produced structural protein (RAFTIN) that is essential for pollen development. Proc Natl Acad Sci 100:14487–14492

Wilson ZA, Zhang D-B (2009) From Arabidopsis to rice: pathways in pollen development. J Exp Bot 60:1479–1492

Xu J, Yang C, Yuan Z, Zhang D, Gondwe MY, Ding Z, Liang W, Zhang D, Wilson ZA (2010) The *ABORTED MICROSPORES* regulatory network is required for postmeiotic male reproductive development in Arabidopsis thaliana. Plant Cell 22:91–107

Yang C, Vizcay-Barrena G, Conner K, Wilson ZA (2007) *MALE STERILITY1* is required for tapetal development and pollen wall biosynthesis. Plant Cell 19:3530–3548

Yeats TH, Rose JK (2008) The biochemistry and biology of extracellular plant lipid-transfer proteins (LTPs). Protein Sci 17:191–198

Zhang D, Wilson ZA (2009) Stamen specification and anther development in rice. Chn Sci Bull 54:2342–2353

Zhang D, Liang W, Yin C, Zong J, Gu F, Zhang D (2010) *OsC6*, encoding a lipid transfer protein, is required for postmeiotic anther development in rice. Plant Physiol 154:149–162

Zhang D, Luo X, Zhu L (2011) Cytological analysis and genetic control of rice anther development. J Genet Genom 38:379–390

Zhao J, Dixon RA (2009) MATE transporters facilitate vacuolar uptake of epicatechin 3′-O-glucoside for proanthocyanidin biosynthesis in Medicago truncatula and Arabidopsis. Plant Cell 21:2323–2340

Zhu L, Shi J, Zhao G, Zhang D, Liang W (2013) *Post-meiotic Deficient Anther1* (*PDA1*) encodes an ABC transporter required for the development of anther cuticle and pollen exine in rice. J Plant Biol 56:59–68

Zinkl GM, Zwiebel BI, Grier DG, Preuss D (1999) Pollen-stigma adhesion in Arabidopsis: a species-specific interaction mediated by lipophilic molecules in the pollen exine. Development 126:5431–5440

Transport of Monoterpenoid Indole Alkaloids in *Catharanthus roseus*

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Abstract Alkaloids are a large and diverse group of natural compounds that are related by the occurrence of a nitrogen atom within a heterocyclic backbone. Due to their strong and divergent biological activities, some of them are applied for clinical uses. Alkaloids are often highly accumulated in particular sites in plants and are often translocated from source tissue/organ to "sink" tissue/organ. Accordingly the complex development-, environment-, organ-, and cell-specific expression of pathway genes include the trafficking of biosynthetic intermediates between different organelles and also their movement between different cell types. Recently the involvement of ABC transporters in alkaloid translocation has been documented thus beginning an interesting new phase in the description of the biochemical components that are required for alkaloid biosynthesis. The identification of these alkaloid transporters is helping to better understand how intra- and inter-cellular compartmentation play important roles in the production/accumulation of alkaloids and supplies new approaches for engineering alkaloid production by manipulating pathway intermediate transport. As an important medicinal plant, *Catharanthus roseus* remains the only source of the anticancer drugs vinblastine and vincristine where it accumulates at very low levels, even if their catharanthine- and vindoline-

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building blocks are much more abundant. The in planta biosynthesis of these compounds is very complex involving several different organelles and cell types. Recently, a new ATP-binding cassette (ABC) transporter, CrTPT2, has been identified that controls catharanthine secretion to leaf surface from leaf epidermis where catharanthine biosynthesis occurs. In this review, we discuss intra- and intercellular compartmentation of the monoterpenoid indole alkaloid (MIA) pathway in *Catharanthus roseus* and TPT2 transporter families possibly involved in MIA secretion in all MIA active plant species.

1 Introduction

Alkaloids are low-molecular weight, nitrogen-containing compounds classified as plant secondary or "special" metabolites that show a wide variety of chemical structures and biological activities. Alkaloids occur in about 20 % of plant species where over 12,000 different structures have been elucidated. The complexity of their chemical structures and their biological activities have made them excellent sources for drug discovery screening programs over the past several decades that have resulted in numerous clinical uses of individual alkaloids (Fester 2010; Croteau et al. 2000). To meet the market demand for many alkaloid drugs, they continue to be sourced from field cultivation of medicinal plants that produce them at low levels where they require expensive extraction and purification procedures for their isolation. The low yield of alkaloid drugs from field grown plants stimulated the development of plant cell, tissue, or organ cultures that might produce high yields of desired compounds since the past 60 years (Zenk et al. 1977; Moreno et al. 1995; Kutchan 1995). With the elucidation of alkaloid biosynthesis pathways, feasible strategies for using cell cultures as vehicles for biotransformation were conceived for engineering alkaloid production in plants and other heterologous hosts (Ziegler and Facchini 2008; Shen et al. 1998; Kutchan 1995).

Some of these alkaloids are very toxic to their original plants, which leads to movement of these compounds from the biosynthetic sites to storage or sink sites to avoid self-poisoning effects (Bird et al. 2003; Samanani et al. 2005; St-Pierre et al. 1999). For example, nicotine is biosynthesized near the apex of roots in *Nicotiana* species, but accumulated in the vacuoles of leaves (Shoji et al. 2000; Hashimoto and Yamada 2003). Berberine is accumulated in the inner bark of *Phellodendron amurense* and caffeine is accumulated in the seeds of *Coffea* spp. as a result of transport from the sites of biosynthesis in other parts of the plant (Shitan and Yazaki 2007; see also chapter "ABC Proteins and Other Transporters in *Lotus japonicus* and *Glycine max*"). In *Catharanthus roseus*, catharanthine is biosynthesized in leaf epidermis but accumulated on the leaf surface together with wax exudates (Roepke et al. 2010). The translocation of catharanthine could serve a dual purpose by increasing plant resistance to herbivores, while decreasing

its toxicity to the plant through its removal from leaf epidermal cells. During the past several decades, both passive and active forms of transport have been proposed for the translocation of alkaloids for biotechnological application (Brodelius and Pedersen 1993; Cai et al. 2012) in view of producing secondary metabolites through exudates in plant cell suspension and hairy root cultures. While many of these studies focused on developing triggering mechanism to promote exudation of secondary metabolites, little progress on the molecular mechanisms involved has been made.

2 Developmental, Environmental, and Spatial Organization of MIA Biosynthesis in *C. roseus*

The Apocynaceae, Loganiaceae, and Rubiaceae plant families are composed of many thousands of plant species responsible for production of several thousand biologically active MIAs that have been identified in nature (Saxton 1997). The majority of all MIAs are derived from the central intermediate, strictosidine that is derived from tryptamine and secologanin precursors (Stöckigt and Zenk 1977; Kutchan 1993; McKnight et al. 1990). The cleavage of strictosidine to a reactive aglycone generates several interconvertible intermediates that can be re-arranged and substituted through a series of oxidations, reductions, and other substitution reactions to generate the diversity of known MIAs (De Luca and Laflamme 2001; Facchini and De Luca 2008; Ziegler and Facchini 2008). The remarkable biological activities of MIAs have led to many clinical applications for treatment of neurological disorders, cardiovascular disease, and cancers in humans (Creasey 1994; Noble 1990).

Among the most important MIA producing plants, *C. roseus* is the only known source of the anticancer drugs, vinblastine and vincristine. The biosynthesis MIAs in *Catharanthus* has been extensively studied through chemical, biochemical, and molecular studies that have led to the identification of over 20 genes that have been functionally characterized for the assembly of the iridoid, secologanin, and MIAs (Salim and De Luca 2013; O'Connor and Maresh 2006; El-Sayed and Verpoorte 2007). Studies with known pathway genes showed that their expression was strictly regulated by development-, environment-, tissue-, and cell-specific controls (De Luca et al. 2012) with the involvement of six intracellular compartments (plastid, chloroplast, vacuole, nucleus, endoplasmic reticulum, and cytosol) and at least three cell types (epidermal cells, internal phloem-associated parenchyma cells, laticifer, and idioblast cells) (St-Pierre et al. 1999; Burlat et al. 2004; De Luca and Cutler 1987; Roepke et al. 2010; Facchini and De Luca 2008; Verma et al. 2012).

2.1 The Intra- and Intercellular Compartmentation of MIA Biosynthesis

The complex compartmentation of MIA biosynthesis and accumulation has created the impetus for discovery of the transporters likely to be involved for shuttling intermediates between organelles and between cells in *C. roseus* leaves. For biosynthesis of tryptamine and secologanin, tryptophan is converted to tryptamine from tryptophan by a single tryptophan decarboxylase gene (TDC) that is expressed in the cytosol (De Luca and Cutler 1987; Stevens et al. 1993) of leaf epidermal cells (St-Pierre et al. 1999), while secologanin is derived from geraniol via eight steps that have now been characterized at the molecular level (De Luca et al. 2014). Geraniol is derived from the methyl erythrytol phosphate pathway (Contin et al. 1998; Oudin et al. 2007) that is preferentially expressed in specialized internal phloem parenchyma (IPAP) cells (reviewed in Salim and De Luca 2013). The assembly of loganic acid from geraniol involves five key steps [geraniol 10-hydroxylase (G10H), Iridodial cyclase (IS), 7-deoxyloganetic acid synthase (7DLS), 7-deoxyloganetic acid glucosyltransferase, (UGT7), and deoxyloganic acid 7-hydroxylase (DL7H)] that also occur exclusively in IPAP cells (Fig. 1) (Irmler et al. 2000; Burlat et al. 2004; Geu-Flores et al. 2012; Asada et al. 2013; Salim et al. 2013; Salim et al. 2014). The G10H, 7DLS and DL7H are Cytochrome P450s associated with the endoplasmic reticulum, which appears to act as a scaffold for the assembly of loganic acid (Fig. 1). The leaf epidermis localized expression of loganic acid methyltransferase (LAMT) (Roepke et al. 2010) and secologanin synthase (SLS) (Irmler et al. 2000) suggests that unknown transport mechanisms for moving loganic acid from IPAP cells to leaf epidermal cells are required in order to complete the biosynthesis of secologanin in epidermal cells (Fig. 1). The strictosidine synthase (STR)-based assembly of strictosidine that has been suggested to occur in plant vacuoles (McKnight et al. 1990, 1991; Guirimand et al. 2010, 2011a, b) of leaf epidermal cells requires tryptamine and secologanin transporters to import these precursors and a strictosidine transporter to export this central intermediate for subsequent reactions (Fig. 1). Cell and molecular studies showed that localize strictosidine β-glucosidase (SGD) to the nucleus (Guirimand et al. 2010) would require the transport of strictosidine into the nucleus and safe export of reactive aglycone intermediates back into the cytosol for further modification into structurally different MIAs like ajmalicine and catharanthine or into tabersonine derivatives required for the final assembly of vindoline (Fig. 1) (St-Pierre et al. 1999; Murata and De Luca 2005; Levac et al. 2008). Upon conversion of tabersonine to 16-methoxytabersonine through the action of tabersonine-16-hydroxylase (T16H) and 16-hydroxytabersonine-16-*O*-methyltransferase (16OMT), the 16-methoxy tabersonine would require oriented transport to specialized idioblast/laticifer cells. While the conversion of 16-methoxytabersonine to 16-methoxy-2,3-dihydro-3-hydroxy- tabersonine occurs through an uncharacterized reaction, transport of an intermediate is required into the chloroplast thylakoid (De Luca and Cutler 1987) for *N*-methylation (NMT) to produce

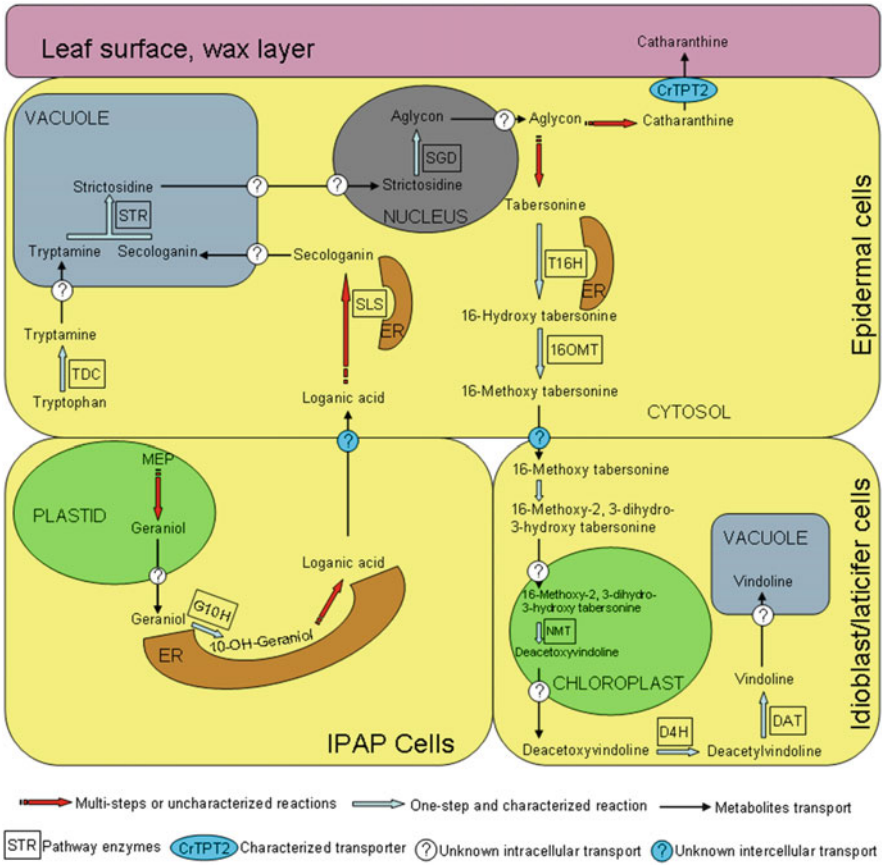

Fig. 1 Proposed compartmentation of MIA pathway and intermediates transport in *Catharanthus* leaves

deacetoxyvindoline (NMT). Transport of this intermediate back to the cytoplasm is then required in order to produce vindoline via deacetoxyvindoline 4-hydroxylase (D4H) and deacetylvindoline 4-O-acetyltransferase (DAT) (Fig. 1) (De Luca et al. 1986; De Carolis et al. 1990; St-Pierre et al. 1998). The vindoline product then requires transport to idioblast/laticifer vacuoles for storage. While no steps required for the assembly of catharanthine are known, we have discovered that this MIA appears to be found as a secretion on the leaf surface together with leaf wax exudates and with ursolic acid that accumulates at high levels (Roepke et al. 2010; Murata et al. 2008). This suggests that another plasma membrane transporter would be responsible for the oriented secretion of catharanthine (Fig. 1, CrTPT2) resulting in the spatial separation of catharanthine and vindoline that helps to explain why only low levels of dimeric MIAs can be found in *C. roseus*.

3 Alkaloid Transport in Plants

Many studies on the organization of alkaloid biosynthesis have documented the involvement of different subcellular compartments and cell types in their assembly (Facchini and De Luca 2008; Ziegler and Facchini 2008). The overwhelming complexity involved includes the need for transport mechanisms that allow movement of intermediates from sites of biosynthesis within or between cells in order to elaborate the complete pathway or to permit accumulation and storage of end products. For example, root-synthesized pyrrolizidine alkaloid *N*-oxides in *Senecio vernalis* are mobilized and allocated throughout the plant via phloem translocation (Hartmann 1999), while in *Nicotiana* species nicotine is translocated via the xylem (Hashimoto and Yamada 1994). This long-distance transport of toxic alkaloids from sites of biosynthesis in the root to peripheral aboveground tissues (reproductive floral meristems and epidermal tissues) are equipped to accumulate these toxic compounds for protecting alkaloid rich plant tissues from the majority of herbivores. The lack of information about the transporters involved within these systems, in *C. roseus* and in most other alkaloid producing plant species makes this an important area of research for understanding the multicell-type coordination and regulation of alkaloid biosynthesis.

3.1 ABC Transporters Involved in Alkaloid Transport

The presence of large numbers of ABC transporters in plants together with numerous reports of mammalian ABC transporters that function as exporters of plant-derived alkaloids raised the possibility that members of this family play key roles in the transport of alkaloids in plants. Plant ABC proteins can be divided into 13 subfamilies according to their sequences and structures. These plant ABC transporters are involved in a large range of common plant activities, such as hormone transport, lipid catabolism, xenobiotic detoxification, disease resistance, stomata function, and also secondary metabolism (Rea 2007; Martinoia et al. 2002). Recently, a number of ABC transporters were reported to be involved in secondary metabolism for transporting these important molecules. Among functionally characterized ABC transporters, CjMDR1, a member of multidrug resistance subfamily of ABC transporters, was isolated from *Coptis japonica* and was shown to mediate the transport of the benzylisoquinoline alkaloid (BIA) berberine into the cells of *Coptis japonica* rhizomes (Shitan et al. 2003). In opium poppy the biosynthesis of BIAs appears to involve the coordinated participation of phloem companion cells that coordinate the biosynthesis of enzymes involved in BIA production, the phloem sieve elements where these enzyme are translocated for BIA biosynthesis and the laticifers where BIAs are stored (Lee et al. 2013). While the complex relationships between these different cell types imply the involvement of alkaloid transporters, little is known about them (Yazaki 2006).

3.2 *Transporters Involved in MIA Biosynthesis in* C. roseus

The search for MIA transporters in *C. roseus* was first investigated at the biochemical level in *Catharanthus* plant cell culture systems in the 1980s when researchers focused on their accumulation in vacuoles. The results obtained were highly controversial with some showing that the transport process was mediated by highly specific carriers (Deus-Neumann and Zenk 1984, 1986; Wink 1993), while others suggested that MIA accumulation occurred by a nonspecific ion trap mechanism (Blom et al. 1991; Wink and Roberts 1998). More recent studies using a tonoplast vesicle system obtained from *Catharanthus* leaves showed that vindoline uptake by *Catharanthus* tonoplasts occurred through a specific H^+/alkaloid antiport system that required a pH gradient generated by an ATPase and/or pyrophosphatase tonoplast pump (Carqueijeiro et al. 2013). These studies provided biochemical support for the presence of active transport-mediated uptake of alkaloids within *Catharanthus* vacuoles rather than the ion trap mechanism. It is hoped that this transporter will soon be characterized at the molecular level, perhaps to show its role in the transport/bioaccumulation of vindoline in idoblast/laticifer vacuoles (Fig. 1).

Recently a novel PDR subfamily of ABC transporter was identified in five MIA producing species (Eurasian *Vinca minor*, African *Tabernamontana elegans*, North American *Amsonia hubrichtii*, Indian *Rauvolfia serpentina*, and South American *Cinchona ledgeriana*) (Yu and De Luca 2013). Previous phylogenetic studies with the sequenced genomes of *Arabidopsis thaliana* and *Oryza sativa* organized the PDR transporters from these species into five subfamilies (I, II, III, IV, and V; Crouzet et al. 2006; Garcia et al. 2004). Phylogenetic analysis of PDR-type ABCG transporters from the MIA-producing species assigned them to subfamily V and particularly to those known to be involved in cuticle assembly in Arabidopsis, rice, and barley (Bessire et al. 2011; Chen et al. 2011; Jasinski et al. 2003). Furthermore, each of these MIA producing species contained two PDR transporters (TPT2 and TPT5) that could be assigned to the subfamily V, while species not known to produce MIAs only had a single PDR transporter that was more closely associated with the gene involved in cuticle assembly. Amino acid sequence analysis showed that CrTPT2 and CrTPT5 are 67.5 % identical. Phylogenetic analysis further divided these cluster V PDRs into two subfamilies, V-A and V-B. Subfamily V-A PDRs are more closely related to AtPDR4, OsPDR6, and HvPDR6 involved in cuticle assembly, while subfamily V-B PDRs are specifically associated with MIA producing plant species (Yu and De Luca 2013). These observations suggest that a gene duplication of ABC transporters involved in cuticle-formation might have taken place and one gene was recruited in MIA-produced plant species to evolve the novel physiological role associated with the secretion of MIAs.

3.3 *The ABC Transporter, CrTPT2, Controls Catharanthine Secretion to the Leaf Surface in* C. roseus

The *Catharanthus* PDR/ABCG transporter CrTPT2 belongs to the pleiotropic drug resistance subfamily of ABC transporters that is the most common ABC transport family in yeast (9 PDRs) and the fifth most common (15 PDRs) in *Arabidopsis* (Rogers et al. 2001; Van den Brûle and Smart 2002). Genes encoding PDR proteins have not been identified in animal or in prokaryotic systems, suggesting that they may play plant-related roles. The first identified PDR genes in plants were *SpTUR2* in *Spirodela polyrrhiza* and *NpPDR1* in *Nicotiana plumbaginifolia*. Both of these transporters appear to participate in the transport of terpenes and presumably other toxic metabolites (Smart and Fleming 1996; Jasinski et al. 2001).

The selection of CrTPT2 as a candidate transporter involved in alkaloids biosynthesis (Yu and De Luca 2013) was based on its preferential expression in in *Catharanthus* leaf epidermis cDNA database (Murata et al. 2008). Further gene expression analysis confirmed its leaf epidermis specific expression and also its preferred expression in younger leaves where the MIA pathway is most active. Treatment of *Catharanthus* leaves with catharanthine or with the methyl jasmonate stress hormone induced the MIA pathway and *CrTPT2* in a coordinate manner (Yu and De Luca 2013).

Functional analysis of CrTPT2 in yeast cells revealed that it functioned as a catharanthine exporter resulting in lower accumulation of catharanthine in *CrTPT2*-expressing cells than in appropriate controls expressing empty vectors. Deletion of two important ATP/GTP binding motifs of CrTPT2 resulted in loss of its catharanthine transport activity, which showed that the functional CrTPT2 required ATP for catharanthine transport. When CrTPT2 was expressed fused to green florescent protein, the transporter was clearly associated with the plasma membranes of yeast and onion epidermal cells. Together these results suggested that CrTPT2 is involved in translocation of catharanthine.

To further clarify the physiological function of CrTPT2 in planta, VIGS (Virus Induced Gene Silencing) was used to knock down the expression of *CrTPT2* in *Catharanthus* leaves. Silenced *CrTPT2*-VIGS leaves showed a 50 % lower expression of *CrTPT2* compared to controls resulting in a 30 % drop in catharanthine levels on the leaf surface and a tenfold increase inside the leaf. While silencing of the transporter did not affect the levels of vindoline, silenced lines accumulated threefold more 3′, 4′-anhydrovinblastine dimer. These results suggest that dimeric MIA production could be enhanced by preventing the export of catharanthine to the leaf surface.

4 Conclusions and Future Prospects

Recent studies have revealed that assembly of MIAs in *C. roseus* is a remarkably dynamic process, involving the participation of at least three cell types within leaves and six organelles inside cells. The precise inter- and intracellular compartmentation of MIA biosynthesis defines the timing and location of MIA biosynthesis as well as where particular MIAs will accumulate. These processes are regulated by tissue-, cell-, and development-specific controls as well as by a number of poorly characterized transporters (Fig. 1). In contrast to the significant progress made with the elucidation of MIA biosynthesis, little is known about the transport of MIA pathway intermediates. So far, only one PDR subfamily of ABC transporter, CrTPT2, has been identified at the molecular level for its catharanthine transport activity (Yu and De Luca 2013) and a vacuolar membrane-associated proton antiport system involved in vindoline transport has been characterized through biochemical means (Carqueijeiro et al. 2013).

The most recent developments in high throughput sequencing of a number of medicinal plant species is providing a rich supply of candidate transporters that are likely to be involved in the transport of alkaloids between and within cells of a given species. These publicly available transcriptomes [Phytometasyn (http://www.phytometasyn.com/; Facchini et al. 2012; Xiao et al. 2013); Medicinal Plant Genomics Consortium (http://www.medicinalplantgenomics.msu.edu/; Góngora-Castillo et al. 2012a, b); Medicinal Plant Transcriptome Project (http://www.uic.edu/pharmacy/MedPlTranscriptome/index.html)] and CATHACyc (http://www.cathacyc.org/; Van Moerkercke et al. 2013) can be mined by many more researchers who are interested in identifying the roles played by alkaloid transporters in the biology, chemical ecology, and evolution of alkaloid pathways in plants. Ultimately, this knowledge should find important applications in metabolic engineering for targeted production of these compounds in host plants or in heterologous hosts such as yeast or in other plants.

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References

Asada K, Salim V, Masada-Atsumi S, Edmunds E, Nagatoshi M, Terasaka K, Mizukami H, De Luca V (2013) A 7-Deoxyloganetic acid glucosyltransferase contributes a key step in secologanin biosynthesis in *Madagascar Periwinkle*. Plant Cell Online 25(10):4123–4134

Bessire M, Borel S, Fabre G, Carraça L, Efremova N, Yephremov A, Cao Y, Jetter R, Jacquat A-C, Métraux J-P (2011) A member of the PLEIOTROPIC DRUG RESISTANCE family of ATP

binding cassette transporters is required for the formation of a functional cuticle in *Arabidopsis*. Plant Cell Online 23(5):1958–1970

Bird DA, Franceschi VR, Facchini PJ (2003) A tale of three cell types: alkaloid biosynthesis is localized to sieve elements in *opium poppy*. Plant Cell Online 15(11):2626–2635

Blom T, Sierra M, Van Vliet T, Franke-van Dijk M, De Koning P, Van Iren F, Verpoorte R, Libbenga K (1991) Uptake and accumulation of ajmalicine into isolated vacuoles of cultured cells of *Catharanthus roseus* (L.) G. Don. and its conversion into serpentine. Planta 183(2): 170–177

Brodelius P, Pedersen H (1993) Increasing secondary metabolite production in plant-cell culture by redirecting transport. Trends Biotechnol 11(1):30–36

Burlat V, Oudin A, Courtois M, Rideau M, St-Pierre B (2004) Co-expression of three MEP pathway genes and geraniol 10-hydroxylase in internal phloem parenchyma of *Catharanthus roseus* implicates multicellular translocation of intermediates during the biosynthesis of monoterpene indole alkaloids and isoprenoid-derived primary metabolites. Plant J 38(1): 131–141

Cai Z, Kastell A, Knorr D, Smetanska I (2012) Exudation: an expanding technique for continuous production and release of secondary metabolites from plant cell suspension and hairy root cultures. Plant Cell Rep 31:461–477

Carqueijeiro I, Noronha H, Duarte P, Gerôs HV, Sottomayor M (2013) Vacuolar transport of the medicinal alkaloids from *Catharanthus roseus* is mediated by a proton driven antiport. Plant Physiol 162:1486–1496

Chen G, Komatsuda T, Ma JF, Nawrath C, Pourkheirandish M, Tagiri A, Hu Y-G, Sameri M, Li X, Zhao X (2011) An ATP-binding cassette subfamily G full transporter is essential for the retention of leaf water in both wild barley and rice. Proc Natl Acad Sci 108(30):12354–12359

Contin A, van der Heijden R, Lefeber AW, Verpoorte R (1998) The iridoid glucoside secologanin is derived from the novel triose phosphate/pyruvate pathway in a *Catharanthus roseus* cell culture. FEBS Lett 434(3):413–416

Creasey WA (1994) Pharmacology, biochemistry, and clinical applications of the monoterpenoid alkaloids. Chem Heterocyclic Comp: Indoles, Part Four, The Monoterpenoid Indole Alkaloids 25:783–829

Croteau R, Kutchan TM, Lewis NG (2000) Natural products (secondary metabolites). In: Biochemistry and molecular biology of plants. pp 1250–1318

Crouzet J, Trombik T, Fraysse ÅS, Boutry M (2006) Organization and function of the plant pleiotropic drug resistance ABC transporter family. FEBS Lett 580(4):1123–1130

De Carolis E, Chan F, Balsevich J, De Luca V (1990) Isolation and characterization of a 2-oxoglutarate dependent dioxygenase involved in the second-to-last step in vindoline biosynthesis. Plant Physiol 94(3):1323–1329

De Luca V, Salim V, Thamm A, Atsumi-Masada S, Yu F (2014) Making iridoids/secoiridoids and monoterpenoid indole alkaloids: progress on pathway elucidation. Curr Opin Plant Biol 19: 35–42

De Luca V, Salim V, Atsumi SM, Yu F (2012) Mining the biodiversity of plants: a revolution in the making. Science 336(6089):1658–1661

De Luca V, Laflamme P (2001) The expanding universe of alkaloid biosynthesis. Curr Opin Plant Biol 4(3):225–233

De Luca V, Cutler AJ (1987) Subcellular localization of enzymes involved in indole alkaloid biosynthesis in *Catharanthus roseus*. Plant Physiol 85(4):1099–1102

De Luca V, Balsevich J, Tyler R, Eilert U, Panchuk B, Kurz W (1986) Biosynthesis of indole alkaloids: developmental regulation of the biosynthetic pathway from tabersonine to vindoline in *Catharanthus roseus*. J Plant Physiol 125(1):147–156

Deus-Neumann B, Zenk M (1984) A highly selective alkaloid uptake system in vacuoles of higher plants. Planta 162(3):250–260

Deus-Neumann B, Zenk M (1986) Accumulation of alkaloids in plant vacuoles does not involve an ion-trap mechanism. Planta 167(1):44–53

El-Sayed M, Verpoorte R (2007) Catharanthus terpenoid indole alkaloids: biosynthesis and regulation. Phytochem Rev 6(2–3):277–305

Facchini PJ, De Luca V (2008) Opium poppy and Madagascar periwinkle: model non-model systems to investigate alkaloid biosynthesis in plants. Plant J 54(4):763–784

Facchini PJ, Bohlmann J, Covello PS, De Luca V, Mahadevan R, Page JE, Ro DK, Sensen CW, Storms R, Martin VJ (2012) Synthetic biosystems for the production of high-value plant metabolites. Trends Biotechnol 30:127–131

Fester K (2010) Plant alkaloids. In: Encyclopedia of life sciences (ELS). Wiley, Chichester. doi:10.1002/9780470015902.a0001914.pub2

Garcia O, Bouige P, Forestier C, Dassa E (2004) Inventory and comparative analysis of rice and *Arabidopsis* ATP-binding cassette (ABC) systems. J Mol Biol 343(1):249–265

Geu-Flores F, Sherden NH, Courdavault V, Burlat V, Glenn WS, Wu C, Nims E, Cui Y, O'Connor SE (2012) An alternative route to cyclic terpenes by reductive cyclization in iridoid biosynthesis. Nature 492(7427):138–142

Góngora-Castillo E, Childs KL, Fedewa G, Hamilton JP, Liscombe DK, Magallanes-Lundback M, Mandadi KK, Nims E, Runguphan W, Vaillancourt B, Varbanova-Herde M, Dellapenna D, McKnight TD, O'Connor S, Buell CR (2012a) Development of transcriptomic resources for interrogating the biosynthesis of monoterpene indole alkaloids in medicinal plant species. PLoS One 7:e52506

Góngora-Castillo E, Fedewa G, Yeo Y, Chappell J, DellaPenna D, Buell CR (2012b) Genomic approaches for interrogating the biochemistry of medicinal plant species. Methods Enzymol 517:139–159

Guirimand G, Guihur A, Ginis O, Poutrain P, Héricourt F, Oudin A, Lanoue A, St-Pierre B, Burlat V, Courdavault V (2011a) The subcellular organization of strictosidine biosynthesis in *Catharanthus roseus* epidermis highlights several trans-tonoplast translocations of intermediate metabolites. FEBS J 278(5):749–763

Guirimand G, Guihur A, Poutrain P, Héricourt F, Mahroug S, St-Pierre B, Burlat V, Courdavault V (2011b) Spatial organization of the vindoline biosynthetic pathway in *Catharanthus roseus*. J Plant Physiol 168(6):549–557

Guirimand G, Courdavault V, Lanoue A, Mahroug S, Guihur A, Blanc N, Giglioli G'h N, St-Pierre B, Burlat V (2010) Strictosidine activation in Apocynaceae: towards a "nuclear time bomb"? BMC Plant Biol 10:182

Hartmann T (1999) Chemical ecology of pyrrolizidine alkaloids. Planta 207(4):483–495

Hashimoto T, Yamada Y (1994) Alkaloid biogenesis: molecular aspects. Ann Rev Plant Biol 45(1):257–285

Hashimoto T, Yamada Y (2003) New genes in alkaloid metabolism and transport. Curr Opin Biotechnol 14(2):163–168

Irmler S, Schröder G, St-Pierre B, Crouch NP, Hotze M, Schmidt J, Strack D, Matern U, Schröder J (2000) Indole alkaloid biosynthesis in *Catharanthus roseus*: new enzyme activities and identification of cytochrome P450 CYP72A1 as secologanin synthase. Plant J 24(6):797–804

Jasinski M, Ducos E, Martinoia E, Boutry M (2003) The ATP-binding cassette transporters: structure, function, and gene family comparison between rice and *Arabidopsis*. Plant Physiol 131(3):1169–1177

Jasinski M, Stukkens Y, Degand H, Purnelle B, Marchand-Brynaert J, Boutry M (2001) A plant plasma membrane ATP binding cassette–type transporter is involved in antifungal terpenoid secretion. Plant Cell 13:1095–1107

Kutchan TM (1993) Strictosidine: from alkaloid to enzyme to gene. Phytochemistry 32(3): 493–506

Kutchan TM (1995) Alkaloid biosynthesis-the basis for metabolic engineering of medicinal plants. Plant Cell 7:1059

Lee E, Hagel J, Facchini P (2013) Role of the phloem in the biochemistry and ecophysiology of benzylisoquinoline alklaoid metabolism. Front Plant Sci 4(182):1–7

Levac D, Murata J, Kim WS, De Luca V (2008) Application of carborundum abrasion for investigating the leaf epidermis: molecular cloning of *Catharanthus roseus* 16-hydroxytabersonine-16-O-methyltransferase. Plant J 53(2):225–236

Martinoia E, Klein M, Geisler M, Bovet L, Forestier C, Kolukisaoglu Ü, Müller-Röber B, Schulz B (2002) Multifunctionality of plant ABC transporters–more than just detoxifiers. Planta 214(3): 345–355

McKnight T, Roessner C, Devagupta R, Scott A, Nessler C (1990) Nucleotide sequence of a cDNA encoding the vacuolar protein strictosidine synthase from *Catharanthus roseus*. Nucleic Acids Res 18(16):4939–4939

McKnight TD, Bergey DR, Burnett RJ, Nessler CL (1991) Expression of enzymatically active and correctly targeted strictosidine synthase in transgenic tobacco plants. Planta 185(2):148–152

Moreno PR, van der Heijden R, Verpoorte R (1995) Cell and tissue cultures of *Catharanthus roseus*: a literature survey. Plant Cell Tissue Organ Cult 42(1):1–25

Murata J, De Luca V (2005) Localization of tabersonine 16-hydroxylase and 16-hydroxytabersonine-16-*O*-methyltransferase to leaf epidermal cells defines them as a major site of precursor biosynthesis in the vindoline pathway in *Catharanthus roseus*. Plant J 44(4):581–594

Murata J, Roepke J, Gordon H, De Luca V (2008) The leaf epidermome of *Catharanthus roseus* reveals its biochemical specialization. Plant Cell Online 20(3):524–542

Noble RL (1990) The discovery of the vinca alkaloids-chemotherapeutic agents against cancer. Biochem Cell Biol 68(12):1344–1351

O'Connor SE, Maresh JJ (2006) Chemistry and biology of monoterpene indole alkaloid biosynthesis. Nat Prod Rep 23(4):532–547

Oudin A, Courtois M, Rideau M, Clastre M (2007) The iridoid pathway in *Catharanthus roseus* alkaloid biosynthesis. Phytochem Rev 6(2–3):259–276

Rea PA (2007) Plant ATP-binding cassette transporters. Annu Rev Plant Biol 58:347–375

Roepke J, Salim V, Wu M, Thamm AM, Murata J, Ploss K, Boland W, De Luca V (2010) Vinca drug components accumulate exclusively in leaf exudates of *Madagascar periwinkle*. Proc Natl Acad Sci 107(34):15287–15292

Rogers B, Decottignies A, Kolaczkowski M, Carvajal E, Balzi E, Goffeau A (2001) The pleitropic drug ABC transporters from *Saccharomyces cerevisiae*. J Mol Microbiol Biotechnol 3(2): 207–214

Salim V, Wiens B, Masada-Atsumi S, Yu F, De Luca V (2014) 7-deoxyloganetic acid synthase catalyzes a key 3 step oxidation to form 7-deoxyloganetic acid in Catharanthus roseus iridoid biosynthesis. Phytochemistry 101:23–31

Salim V, De Luca V (2013) Towards complete elucidation of monoterpene indole alkaloid biosynthesis pathway: *Catharanthus roseus* as a pioneer system. Adv Bot Res 68:1–37

Salim V, Yu F, Altarejos J, Luca V (2013) Virus-induced gene silencing identifies *Catharanthus roseus* 7-deoxyloganic acid-7-hydroxylase, a step in iridoid and monoterpene indole alkaloid biosynthesis. Plant J 76(5):754–765

Samanani N, Park S-U, Facchini PJ (2005) Cell type–specific localization of transcripts encoding nine consecutive enzymes involved in protoberberine alkaloid biosynthesis. Plant Cell Online 17(3):915–926

Saxton JE (1997) Recent progress in the chemistry of the monoterpenoid indole alkaloids. Nat Prod Rep 14(6):559–590

Shen Z, Eisenreich W, Kutchan TM (1998) Bacterial biotransformation of 3α(S)-Strictosidine to the monoterpenoid indole alkaloid vallesiachotamine. Phytochemistry 48(2):293–296

Shitan N, Bazin I, Dan K, Obata K, Kigawa K, Ueda K, Sato F, Forestier C, Yazaki K (2003) Involvement of CjMDR1, a plant multidrug-resistance-type ATP-binding cassette protein, in alkaloid transport in *Coptis japonica*. Proc Natl Acad Sci 100(2):751–756

Shitan N, Yazaki K (2007) Accumulation and membrane transport of plant alkaloids. Curr Pharm Biotechnol 8(4):244–252

Shoji T, Yamada Y, Hashimoto T (2000) Jasmonate induction of putrescine N-methyltransferase genes in the root of *Nicotiana sylvestris*. Plant Cell Physiol 41(7):831–839

Smart CC, Fleming AJ (1996) Hormonal and environmental regulation of a plant PDR5-like ABC transporter. J Biol Chem 271(32):19351–19357

St-Pierre B, Vazquez-Flota FA, De Luca V (1999) Multicellular compartmentation of *Catharanthus roseus* alkaloid biosynthesis predicts intercellular translocation of a pathway intermediate. Plant Cell Online 11(5):887–900

St-Pierre B, Laflamme P, Alarco AM, Luca E (1998) The terminal O-acetyltransferase involved in vindoline biosynthesis defines a new class of proteins responsible for coenzyme A-dependent acyl transfer. Plant J 14(6):703–713

Stevens LH, Blom TJ, Verpoorte R (1993) Subcellular localization of tryptophan decarboxylase, strictosidine synthase and strictosidine glucosidase in suspension cultured cells of *Catharanthus roseus* and *Tabernaemontana divaricata*. Plant Cell Rep 12(10):573–576

Stöckigt J, Zenk MH (1977) Strictosidine (isovincoside): the key intermediate in the biosynthesis of monoterpenoid indole alkaloids. J Chem Soc, Chem Commun 18:646–648

Van Moerkercke A, Fabris M, Pollier J, Baart GJ, Rombauts S, Hasnain G, Rischer H, Memelink J, Oksman-Caldentey KM, Goossens A (2013) CathaCyc, a metabolic pathway database built from *Catharanthus roseus* RNA-Seq data. Plant Cell Physiol 54:673–685

Van den Brûle S, Smart CC (2002) The plant PDR family of ABC transporters. Planta 216(1): 95–106

Verma P, Mathur AK, Srivastava A, Mathur A (2012) Emerging trends in research on spatial and temporal organization of terpenoid indole alkaloid pathway in *Catharanthus roseus*: a literature update. Protoplasma 249(2):255–268

Wink M (1993) The plant vacuole: a multifunctional compartment. J Exp Bot 44:231–231

Wink M, Roberts MF (1998) Compartmentation of alkaloid synthesis, transport, and storage. In: Alkaloids. Springer, Heidelberg, pp 239–262

Xiao M, Zhang Y, Chen X, Lee EJ, Barber CJ, Chakrabarty R, Desgagné-Penix I, Haslam TM, Kim YB, Liu E, MacNevin G, Masada-Atsumi S, Reed D, Stout JM, Zerbe P, Zhang Y, Bohlmann J, Covello PS, De Luca V, Page JE, Ro DK, Martin VJJ, Facchini PJ, Sensen CW (2013) Transcriptome analysis based on next-generation sequencing of non-model plants producing specialized metabolites of biotechnological interest. J Biotechnol 166:122–134

Yazaki K (2006) ABC transporters involved in the transport of plant secondary metabolites. FEBS Lett 580(4):1183–1191

Yu F, De Luca V (2013) ATP-binding cassette transporter controls leaf surface secretion of anticancer drug components in *Catharanthus roseus*. Proc Natl Acad Sci 110(39): 15830–15835

Zenk M, El-Shagi H, Arens H, Stöckigt J, Weiler E, Deus B (1977) Formation of the indole alkaloids serpentine and ajmalicine in cell suspension cultures of *Catharanthus roseus*. In: Plant tissue culture and its bio-technological application. Springer, pp 27–43

Ziegler J, Facchini PJ (2008) Alkaloid biosynthesis: metabolism and trafficking. Annu Rev Plant Biol 59:735–769

Plant Peroxisomal ABC Transporters: Flexible and Unusual

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Abstract ABC transporters of subfamily D mediate import of substrates for β-oxidation into peroxisomes. Whilst mammals possess three peroxisomal ABCD proteins which homodimerise to form transporters with distinct substrate specificities, Baker's yeast has a single transporter formed by heterodimerisation, which imports long-chain fatty acyl CoAs. Plants have a single-fused heterodimer transporter that exhibits broad substrate specificity, reflecting the wide range of β-oxidation substrates processed by plants. The fusion appears to have occurred early in the evolution of land plants and was followed by an early duplication event in the monocot lineage. Plant ABCD proteins function in all stages of the life cycle and their physiological roles reflect the ability to transport diverse substrates including saturated and unsaturated fatty acids and aromatic compounds such as precursors of hormones and secondary metabolites. Recent work suggests that transport of CoA substrates involves their cleavage and re-esterification within the peroxisome, thus interaction with appropriate acyl adenylate-activating enzymes potentially provides a mechanism for regulating entry of different substrates into β-oxidation. The mechanism of ABCD transporter targeting is broadly conserved across kingdoms but evidence suggests the regulation of protein turnover differs.

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Abbreviations

ABA	Abscisic acid
ABC	ATP-binding cassette
ABCD	ABC transporter, subfamily D
ALDP	Adrenoleukodystrophy protein
ALDR	Adrenoleukodystrophy-related protein
CoA	Coenzyme A
CTS	Comatose
2,4-DB	2,4-Dichlorophenoxybutyrate
ER	Endoplasmic reticulum
IAA	Indole acetic acid
IBA	Indole butyric acid
JA	Jasmonic acid
LACS	Long-chain fatty acyl CoA synthetase
LCFA	Long-chain fatty acid
MCFA	Medium chain fatty acid
NBD	Nucleotide-binding domain
OPDA	12-Oxophytodienoic acid
PMP	Peroxisomal membrane protein
PMP69	69 kDa PMP
PMP70	70 kDa PMP
TAG	Triacylglycerol
TMD	Transmembrane domain
VLACS	Very long-chain fatty acyl CoA synthetase
VLCFA	Very long-chain fatty acid
X-ALD	X-linked adrenoleukodystrophy

1 Introduction and Historical Background

Peroxisomes are single-membrane-bound organelles which display diverse and flexible metabolic activities, often sharing metabolic pathways with other compartments and exhibiting functional specialisation in different tissues and organisms (Hu et al. 2012). Unlike mitochondria and chloroplasts, peroxisomes do not contain DNA and must import their constituent proteins post-translationally (Girzalsky et al. 2010; Ma et al. 2011). The protein complement of plant peroxisomes changes during development and in response to prevailing conditions, providing considerable metabolic versatility, although fatty acid β-oxidation and hydrogen peroxide metabolism are common to most peroxisome types. Several novel functions have been ascribed to these organelles by recent proteomic analyses (Reumann et al. 2007, 2009; Arai et al. 2008; Eubel et al. 2008). Plant peroxisomes not only play major roles in the glyoxylate cycle and photorespiration but also contribute to biosynthesis of hormones, isoprenoids, biotin, phylloquinone and volatile

benzenoids as well as housing reactions required for sulphite oxidation and catabolism of polyamines (Hu et al. 2012; Linka and Theodoulou 2013). Several studies have also implicated peroxisomes in pathogen responses (Taler et al. 2004; Lipka et al. 2005; Bednarek et al. 2009; Clay et al. 2009). This functional diversity requires a portfolio of transport activities which serve to import substrates, co-substrates and cofactors, to exchange metabolites and to export products.

Although transport processes are essential for co-ordination of peroxisomal metabolism with that of other subcellular compartments, the permeability of the peroxisome membrane has been hotly debated in the literature. A handful of specific transporters has been discovered but it is not yet clear to what extent these account for the many transport steps which are required for to peroxisomes to fulfil their diverse functions (reviewed in Theodoulou et al. 2011; Antonenkov and Hiltunen 2012; Linka and Theodoulou 2013). Amongst the best studied peroxisomal transporters are the ATP-Binding Cassette (ABC) transporters which mediate import of substrates for β-oxidation. These proteins are the subject of this chapter.

1.1 ABC Transporter Subfamily D and the Discovery of Adrenoleukodystrophy Protein

The importance of peroxisomes is apparent from the severe, sometimes embryolethal phenotypes of plants deficient in key peroxisomal proteins (Sparkes et al. 2003; Fan et al. 2005; Nito et al. 2007) and the existence of human diseases caused by defective peroxisomal metabolism or assembly (Wanders 2004). One such disease, X-linked adrenoleukodystrophy (X-ALD), is an inherited metabolic storage disorder characterised by impaired peroxisomal β-oxidation and the accumulation of very-long chain fatty acids (VLCFAs) in tissues and plasma (Kemp et al. 2011, 2012). In 1993, the primary genetic cause of this disease was found to be mutation of the *ABCD1* gene, which encodes an ABC transporter known as adrenoleukodystrophy protein (ALDP; Mosser et al. 1993). This discovery was greeted with surprise in the peroxisome community, since X-ALD peroxisomes are unable to activate VLCFA to VLCFA-CoA esters and it had been assumed that the defective gene would encode a peroxisomal acyl CoA synthetase (Lazo et al. 1988; Wanders et al. 1988a, b). However, recent studies with inhibitory antibodies have linked the defect in VLCFA β-oxidation directly to transport (Wiesinger et al. 2013).

ALDP belongs to Subfamily D of the ABC transporter superfamily and has homologues in both eukaryotic and prokaryotic taxa. Mammalian genomes have four *ABCD* genes designated *ABCD1-4*, encoding ALDP, ALD-related protein (ALDR), PMP70 and PMP69, respectively (Kemp et al. 2011; Morita and Imanaka 2012). ALDP, ALDR and PMP70 are all peroxisomal proteins with roles in import of different lipid-related substrates (Kemp et al. 2011; van Roermund et al. 2011)

but PMP69 has recently been shown to localise to ER and lysosomes and mutations in *ABCD4* have been unequivocally linked with inborn errors in vitamin B12 (cobalamin) metabolism (Kashiwayama et al. 2009; Coelho et al. 2012). In agreement with this apparent divergence in function, human ABCD4 (HsABCD4) occupies a distinct clade in a subfamily D phylogenetic tree (Fig. 1).

All mammalian ABCD genes encode so-called half size ABC transporters with one transmembrane domain (TMD) preceding a nucleotide-binding domain (NBD). Thus the proteins are expected to dimerise to form functional transporters. Early literature proposed that human peroxisomal ABC transporters might heterodimerise, by analogy to the *Drosophila* subfamily G eye pigment transporters, and that heterodimerisation could serve to increase functional versatility (Theodoulou et al. 2006). However, evidence from a range of sources supports the notion that the transporters are predominantly if not exclusively homodimeric (Guimarães et al. 2004; Wanders et al. 2007; van Roermund et al. 2008; Kemp et al. 2011). Nevertheless, it is clear that heterodimerisation can occur, particularly when transporters are over-expressed, and this is a potentially important consideration for therapeutic approaches to X-ALD employing gene therapy or pharmacological induction of transporters (Theodoulou et al. 2006).

1.2 The ABCD Subfamily in Fungi

Baker's yeast (*Saccharomyces cerevisiae*) has two "half-size" ABCD proteins, Pxa1p and Pxa2p. First identified as ALDP homologues, they were subsequently shown to encode two components of a heterodimeric transporter required for β-oxidation of long-chain (LC) FA (Hettema et al. 1996; Shani and Valle 1996). Homologues are required for utilisation of hydrophobic substrates such as triglycerides and alkanes in the oleaginous yeast, *Yarrowia lipolytica* (Thevenieau et al. 2007) but surprisingly, were found to be dispensable for peroxisomal LCFA import in the filamentous fungus, *Podospora anserina* (Boisnard et al. 2009). The majority of fungi do not have obvious *ABCD4* homologues.

1.3 The ABCD Subfamily in Plants

The ABC transporters of land plant peroxisomes are unusual in that they represent "fused heterodimers", in which an entire transporter protomer is encoded in a single gene (Fig. 2). Arabidopsis has a single such gene, *AtABCD1* or *COMATOSE* (*CTS*, also known as *AtPXA1*, *PED3* and *ACN2*) (Zolman et al. 2001; Footitt et al. 2002; Hayashi et al. 2002; Hooks et al. 2007; Verrier et al. 2008), but most sequenced monocot genomes appear to contain two homologues, suggesting that an early duplication event occurred following the divergence of grass lineage (Verrier et al. 2008; Nyathi et al. 2012; Mendiondo et al. 2014). Moreover, the N-terminal

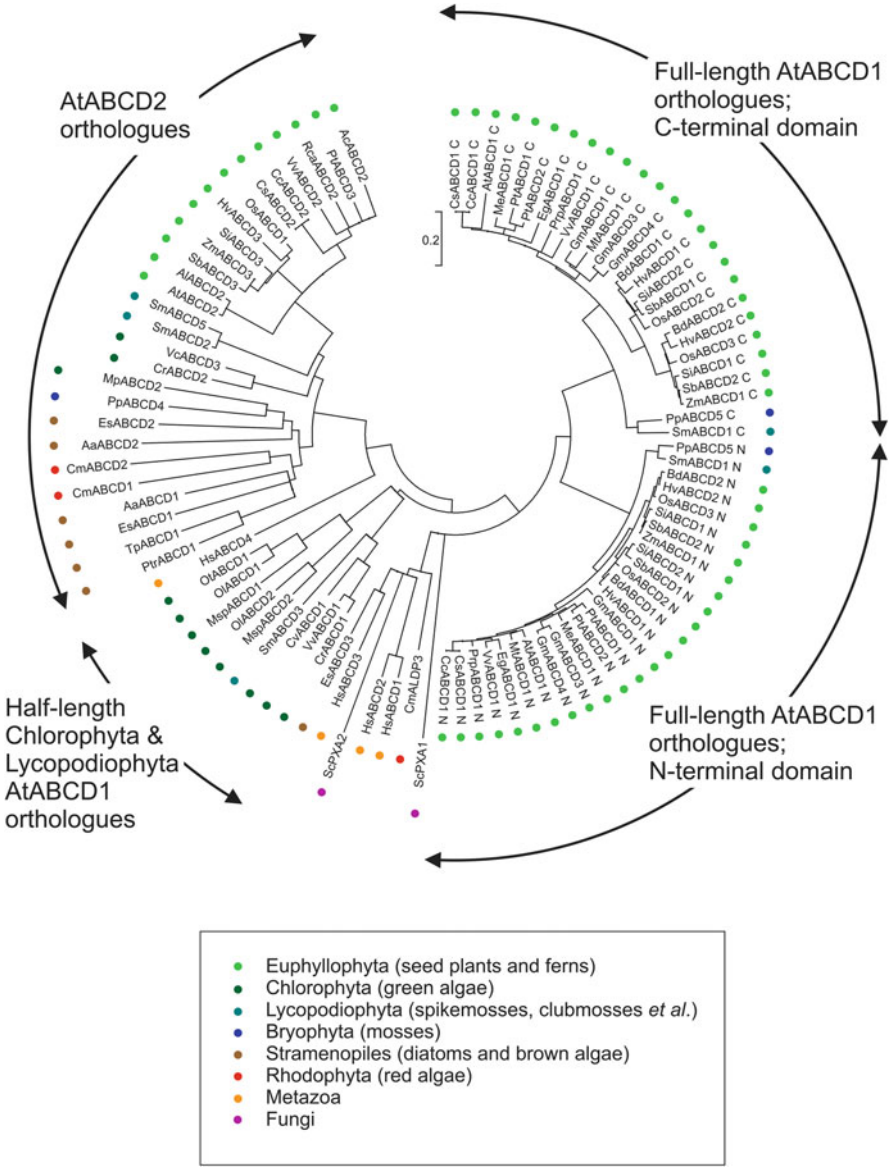


Fig. 1 Phylogenetic analysis of ABC family D transporters. Relationships were analysed by the Maximum Likelihood method. *Upper* and *lower case letters* at the beginning of protein names indicate abbreviated Latin binomial names for species. Subfamily members within each species are classified according to Verrier et al. 2008. Reproduced from Nyathi et al. (2012)

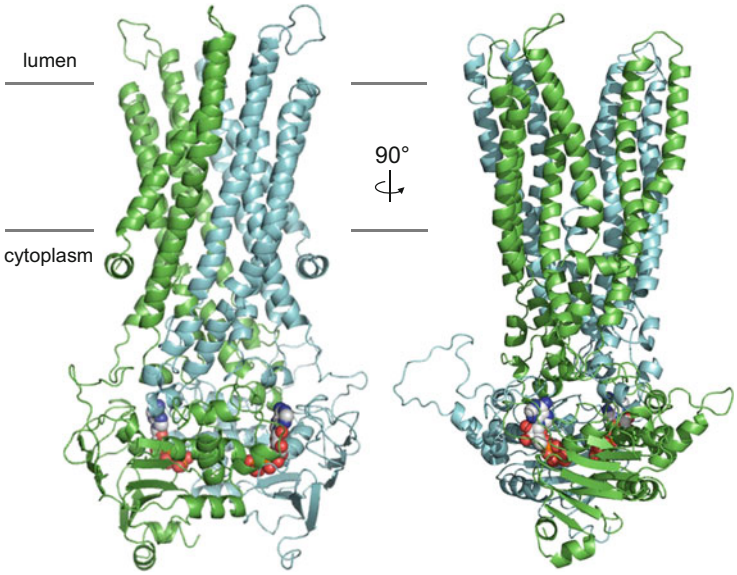


Fig. 2 Homology model of CTS showing major structural features. CTS was modelled on the structure of the bacterial multidrug transporter Sav1866 complexed with ADP (PDB accession 2HYD). AMP-PNP (space-filling representation) has been shown within the NBDs at positions equivalent to those found in the AMPPNP-bound form of Sav1866 (PDB accession 2ONJ) to illustrate the putative location of bound nucleotide. The N- and C-terminal halves of the CTS sequence are shown in *green* and *blue*, respectively. *Grey lines* show the approximate position of the lipid bilayer. *Left* hand panel reproduced from Dietrich et al. (2009)

halves of all "full-size" plant ABCD proteins are more similar to each other than to the C-terminal halves, consistent with the notion that the fusion event only happened once in plant evolution. Algae lack fused ABCD proteins but together with lycophytes, have "half-length" CTS homologues, which appear to form a basal clade and may be ancestral to land plant "full-length" ABCD transporters. Plant genomes from algae to angiosperms also encode a distinct class of "half size" ABCD proteins (AtABCD2 in Arabidopsis) which cluster with PMP69/ABCD4 (Fig. 1). AtABCD2 is a plastid protein and, interestingly, has homologues in cyanobacteria, but it is not clear whether or not it originated from the endosymbiont ancestor of plastids or whether it has a more complex evolutionary history (Tyra et al. 2007). Since plants do not synthesise or use cobalamin (Croft et al. 2005), it seems likely that AtABCD2 and its plant homologues carry out a different function, which has yet to be determined but is likely to be associated with photosynthesis.

Table 1 Physiological and biochemical functions of plant peroxisomal ABC transporters

Developmental or physiological function	Biochemical role of CTS	References
Seed size determination	Not known	Russell (1998), Orsi and Tanksley (2009), Mendiondo et al. (2014)
Germination completion	Removal of OPDA	Footitt et al. (2002, 2006), Pinfield-Wells et al. (2005), Kanai et al. (2010), Dave et al. (2011)
Seedling establishment	Mobilisation of stored TAG	Zolman et al. (2001), Footitt et al. (2002), Hayashi et al. (2002)
Fertilisation (a) pollen tube growth; (b) stamen filament elongation	(a) Mobilisation of stored lipid? (b) IAA synthesis	Footitt et al. (2007)
Survival during dark-induced starvation	Mobilisation of membrane lipids	Kunz et al. (2009a, b), Slocombe et al. (2009)
Wound response; pathogen response?	JA biosynthesis	Theodoulou et al. (2005)
Lateral root development, root hair and cotyledon expansion, temperature-induced hypocotyl elongation, apical hook curvature	IAA synthesis	Zolman et al. (2001), Strader et al. (2010, 2011)
Herbicide metabolism	2,4-DB metabolism	Hayashi et al. (2002)
Cysteine biosynthesis and protein deacetylation?	Acetate reassimilation	Hooks et al. (2007)
Interactions with pests, pathogens and symbionts	Biosynthesis of benzoic acid-derived secondary metabolites (benzoylated glucosinolates, substituted hydroxybenzoyl cholines, volatile benzenoids and salicylic acid)	Bussell et al. (2013)

2 Functions of Plant Peroxisomal ABC Transporters

Our current knowledge of plant peroxisomal ABC transporter function has largely been obtained from characterisation of Arabidopsis mutants, in part because *CTS* (AtABCD1) was identified in at least five independent forward genetic screens, each affording different insights into its biochemical and physiological functions (reviewed in Theodoulou et al. 2011). Taken together, the available evidence strongly supports a model in which CTS is a broad specificity transporter which serves to import the diverse range of substrates which are processed by β-oxidation in plants. In the majority of cases, the different physiological/developmental roles of CTS can be linked to β-oxidation of specific substrates (Table 1).

2.1 Germination and Seedling Establishment

CTS takes its name from its germination phenotype: null mutants were originally thought to be "forever dormant" but physiological assays and transcriptome analysis subsequently revealed that they are arrested late in phase II of germination (Russell et al. 2000; Footitt et al. 2002, 2006; Carrera et al. 2007). The block in germination can be overcome by mechanically disrupting the seed coat and supplying sucrose. CTS is also required for seedling establishment in Arabidopsis: mutants are unable to mobilise seed triacylglycerol (TAG) reserves and consequently retain oil bodies, fatty acids and fatty acyl-CoAs in comparison to wild-type seedlings (Zolman et al. 2001; Footitt et al. 2002; Hayashi et al. 2002). In agreement with this, CTS complements the yeast *pxa1/2Δ* double mutant for oleate utilisation (Nyathi et al. 2010). The biochemical function of CTS in germination is distinct from that in seedling establishment since mutants lacking the lipases or acyl-CoA synthetases required for storage oil mobilisation germinate normally (Pinfield-Wells et al. 2005; Footitt et al. 2006; Kelly et al. 2011). Genetic analysis and hormone profiling demonstrated that CTS functions to reduce levels of the jasmonic acid precursor, oxophytodienoic acid (OPDA), which represses germination through regulation of the *ABSCISIC ACID INSENSITIVE 5* (*ABI5*) transcription factor (Dave et al. 2011). ABI5 inhibits germination in part through control of polygalacturonase-inhibiting proteins which prevent degradation of seed coat pectin (Kanai et al. 2010).

2.2 Post-germinative Functions

Once seedlings have become photosynthetic, *cts* mutants can complete a life cycle but have reduced fertility (Footitt et al. 2007). Although oxylipin profiling revealed that levels of basal and wound-induced jasmonic acid (JA) are reduced but not abolished in leaves of *cts* mutants (Theodoulou et al. 2005), fertility could not be restored by JA treatment of flower buds (Footitt et al. 2007). CTS appears to play more than one role in fertilisation since mutants exhibit reduced stamen filament elongation and pollen tube growth, effects which could be reversed by application of auxin and sucrose, respectively (Footitt et al. 2007). Seed size is reduced in *cts* mutants (Russell 1998) and a CTS homologue, *Seed weight 4.1* (*Sw4.1*) has been identified in an analysis of quantitative trait loci affecting seed size in tomato (Orsi and Tanksley 2009). The precise biochemical function of peroxisomal ABC transporters has not yet been determined in this developmental context but reciprocal crosses show that *Sw4.1* controls seed weight through zygotic effects (Orsi and Tanksley 2009).

CTS expression is low in leaves and although β-oxidation does not play a prominent role in post-germinative vegetative growth, the pathway is up-regulated during senescence and has been postulated to play a role in recycling

of membrane lipids (Buchanan-Wollaston et al. 2005; van der Graaff et al. 2006; Kunz et al. 2009a, b). This has been shown to occur in plants under dark-induced starvation, where mobilisation of lipids provides energy once starch is depleted (Kunz et al. 2009a, b). Ablation of CTS function permits membrane-derived fatty acids to be redirected to TAG synthesis when the LEC2 transcription factor is ectopically expressed in leaves (Slocombe et al. 2009). However, in naturally senescing leaves, fatty acid levels in mutants deficient in enzymes of β-oxidation are similar to those of wild-type plants, arguing against a role in lipid remobilisation, although *cts* alleles were not investigated in this study and it is possible that this result was confounded by functional redundancy (Yang and Ohlrogge 2009).

2.3 β-Oxidation of Auxins

Mutants allelic to *cts* were obtained in screens designed to identify genes involved in the metabolism of the natural auxin, indole butyric acid (IBA) and the auxin analogue, 2,4-dichlorophenoxybutyric acid (2,4-DB), implicating CTS in import of these compounds into peroxisomes (Zolman et al. 2001; Hayashi et al. 2002). Although production of indole acetic acid (IAA) from IBA was not initially considered to be a major biosynthetic route, β-oxidation has since been shown to provide a significant source of IAA in seedlings, playing roles in lateral root development, expansion of root hairs and cotyledons and also contributing to stamen filament elongation in flowers (Zolman et al. 2001; Footitt et al. 2007; Strader et al. 2010, 2011; Theodoulou et al. 2011).

2.4 Other Substrates

The broad specificity of CTS, together with the discovery of new peroxisome functions, points to additional potential substrates (Linka and Theodoulou 2013). Indeed, it has been shown recently that CTS is required for peroxisomal synthesis of benzoic acid (BA) and thus plays a role in biosynthesis of salicylic acid, benzoylated glucosinolates and presumably also volatile benzenoids (Bussell et al. 2014; Qualley et al. 2012). CTS has also been found to function in acetate metabolism since mutant alleles were isolated in a screen for resistance to fluoroacetate (Hooks et al. 2007).

2.5 Comparisons with Non-plant Species

In plants, β-oxidation is confined to the peroxisomes where fatty acids are oxidised to completion and a wide range of other compounds are processed by this pathway (Linka and Theodoulou 2013). Taken together, the available data suggest that CTS is a transporter with relatively broad substrate specificity, which can handle not only fatty acids of different chain lengths and degrees of unsaturation but also smaller, aromatic compounds and acetate. The absence of any other homologues in Arabidopsis and the dramatic phenotypes of *cts* mutants suggest that this transporter plays a major role in importing all of the diverse substrates for β-oxidation, although a minor alternative pathway for some compounds, such as OPDA, cannot be ruled out (Theodoulou et al. 2005). In contrast, mammalian ABCD transporters appear to have much more restricted substrate specificity, each accepting a distinct but overlapping group of compounds (Kemp et al. 2011). This may reflect the different subcellular localisations and roles of mammalian β-oxidation, which is initiated by chain-shortening of VLCFA in peroxisomes and completed in mitochondria following transfer of intermediates. Mammalian peroxisomes also catalyse synthesis of plasmalogens, production of C22:6 VLCFA from C24:6 and catabolism of bile acids (Wanders et al. 2007). ALDP and ALDR preferentially handle saturated and polyunsaturated VLCFA, respectively, and PMP70 appears to be important for β-oxidation of pristanic and dicarboxylic acids (van Roermund et al. 2008, 2011, 2013; Genin et al. 2011; Kemp et al. 2011). In agreement with this, neither ALDP nor ALDR could complement the Arabidopsis *cts-1* mutant, despite being correctly targeted to peroxisomes (Zhang et al. 2011). β-oxidation is also solely peroxisomal in yeast. Genetic experiments have shown that Pxa1p/2p can mediate import of FA with chain lengths from C14 to C24 but in vivo, medium chain fatty acids can cross the peroxisome membrane independently of Pxa1p/2p and are activated in the peroxisome lumen by the acyl-CoA synthetase, Faa2p (Hettema et al. 1996; Wanders et al. 2007; van Roermund et al. 2008). It is not known whether the yeast transporter is also capable of handling aromatic substrates.

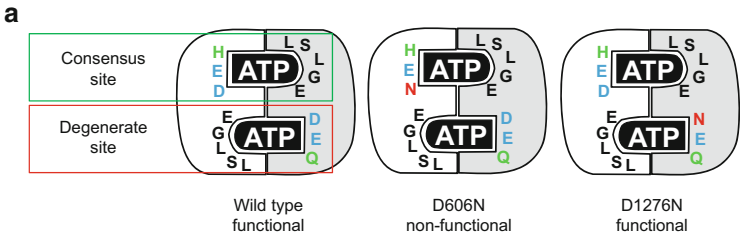
3 Structure, Mechanism and Regulation

Although much is now known about the diversity of peroxisomal ABC transporter functions, information is lacking regarding structure-function relationships. The precise identity of the substrates and their mechanism of translocation across the membrane have been matters of much debate but recent advances have provided insight into these issues and suggested a mechanism which contributes to control of peroxisomal metabolism.

3.1 Structural and Catalytic Asymmetry

The CTS protein has the topology TMD-NBD-TMD-NBD, with the two transmembrane domains each containing six α-helices which are predicted to extend beyond the lipid bilayer into the cytosol (Dietrich et al. 2009). Homology modelling using the bacterial Sav1866 crystal structure as a template (Dawson and Locher 2006) suggests that the folded molecule is "domain-swapped", such that helices 1,2,9,10,11 and 12 form one wing of the transporter and helices 3–8 the other. Wing A (mainly consisting of helices from TMD1) contacts NBD2 and wing B contacts NBD1 (Fig. 2; Dietrich et al. 2009). "Transmission interfaces", involved in coupling ATP binding and hydrolysis to transmembrane movement of solutes are formed where the transmembrane domains contact the nucleotide binding domains (Dawson and Locher 2006).

Activity of ABC transporters requires the formation of an NBD sandwich dimer, which contains two composite nucleotide-binding sites (Smith et al. 2002; Moody et al. 2002; Figs. 2 and 3). Each nucleotide-binding site is composed of the conserved Walker A, Walker B and H-loop regions of one NBD and the signature motif of the other. Thus, whilst homodimeric proteins such as ALDP have two identical nucleotide-binding sites, heterodimeric or pseudoheterodimeric ABC transporters such as Pxa1p/Pxa2p and CTS have dissimilar binding sites. Additionally, Pxa1p and the C-terminal NBD of CTS have amino acid substitutions at conserved positions within the composite binding site, which predict that one site will be catalytically impaired (Fig. 3; Dietrich et al. 2009). This asymmetry is relatively common in the ABC transporter superfamily and has apparently evolved several times, since some subfamilies contain both symmetrical and asymmetrical transporters (Procko et al. 2009). Analysis of site-directed mutants in planta has demonstrated that the two NBDs of CTS are indeed functionally non-equivalent: mutation of Walker A and B motifs in NBD1 led to a complete loss-of-function phenotype, whereas plants bearing the equivalent mutations in NBD2 appeared wild type (Dietrich et al. 2009). Moreover, function was affected by mutation of a conserved glutamate residue in the predicted "transmission helix" contacting NBD1 but not in the equivalent residue contacting NBD2. Nevertheless, both N- and C-terminal halves are required for activity, as shown by co-expression of CTS pseudo-half molecules in yeast (Fig. 3; Nyathi et al. 2012). Whilst hydrolysis of a single ATP molecule is evidently sufficient for transport activity, the role of nucleotide binding at the non-hydrolytic site and the detailed implications for the catalytic cycle of CTS and other asymmetric ABC transporters remain to be determined (Fig. 4).



b

	Walker A	Signature	Walker B *	H-loop
CTS NBD1	GPNGSGKSS	ELSLGEQQ	FAILDE	SHRP
CTS NBD2	GPNGSGKTS	ILSLGEQQ	FGVLDE	SQRP
ABCD1	GPNGCGKSS	VLSGGEKQ	YALLDE	THRP
ABCD2	GPNGCGKSS	VLSGGEKQ	YALLDE	THRP
ABCD3	GPNGCGKSS	VLSGGEKQ	FAILDE	SHRK
Pxa1p	GPNGCGKSS	LLSGGEKQ	YVVLDE	SQRP
Pxa2p	GPNGCGKSS	ELTIGVQQ	FAVLDE	CHRT
TAP1	GPNGSGKST	QLSGGQRQ	VLILDD	TQHL
TAP2	GPNGSGKST	QLAAGQKQ	VLILDE	AHR

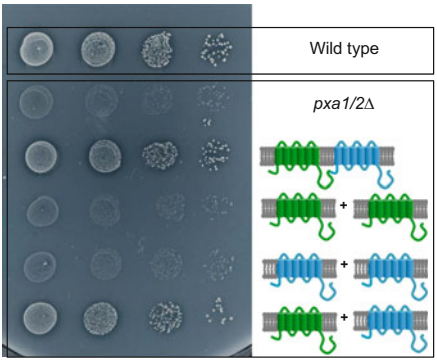


Fig. 3 Symmetry and asymmetry in ABCD transporters. (**a**) Schematic diagrams of CTS NBD dimers for wild type and the D606N and D1276N mutants. NBD1 (*white*) and NBD2 (*grey*) form two nucleotide (*black*)-binding sites. Each nucleotide-binding site is composed of the Walker A, Walker B, and H-loop of one NBD and the signature motif of the other, but the Walker A motif is omitted for clarity. The upper ATP-binding site has the consensus sequence, whereas the lower site is degenerate (Q instead of H in H-loop). (**b**) Alignment of conserved NBD motifs of Arabidopsis CTS, *S. cerevisiae* Pxa1p, Pxa2p, human ABCD1, ABCD2, ABCD3 and human TAP1/2 (ABCB subfamily). Amino acids shown in (**a**) are colour-coded, with the mutated Walker B aspartate residue indicated by an asterisk. (**c**) Growth on solid oleate medium of wild-type and mutant (*pxa1/2Δ*) yeast cells transformed with full-length CTS and different combinations of CTS pseudo-half molecules. The clear haloes around the colonies indicate utilisation of oleate. **a**,**b** redrawn from Dietrich et al. (2009); **c** modified from Nyathi et al. (2012)

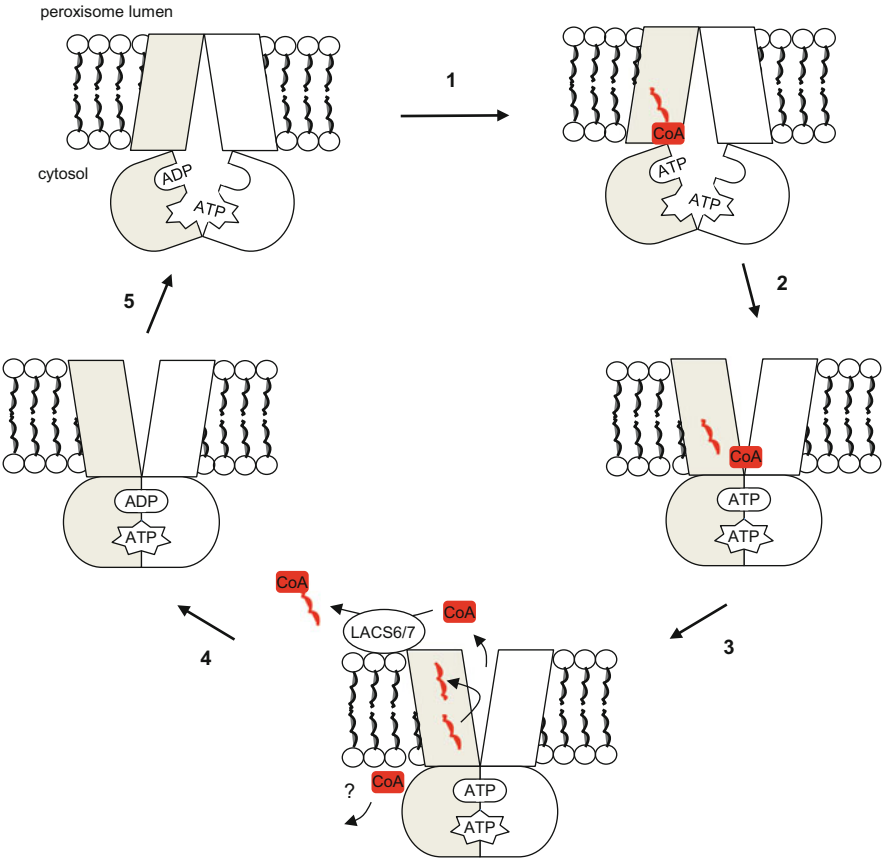


Fig. 4 A hypothetical transport mechanism for CTS. Diagram based on a general mechanism for asymmetric ABC transporters (Procko et al. 2009). In the resting state, nucleotide-binding domains (NBDs) are open and the transporter is in the inward (cytosol)-facing conformation. 1. Substrate binding to transmembrane domains (TMDs) and ADP/ATP exchange in NBD1. The amphipathic acyl-CoA substrate (depicted in *red*) may access the TMDs from the lipid bilayer. 2. NBD closure triggered by ATP binding, accompanied by change in conformation to the outward (lumen)-facing state. Thioesterase activity releases the CoA moiety. 3. CoA moiety exits to the lumenal side of the membrane, via the hydrophilic central chamber and the fatty acid moiety flip-flops in the lipid bilayer. Fatty acid is re-esterified to CoA by peroxisomal acyl-CoA synthetases, LACS6/7, which interact physically with CTS. Alternatively, CoA could be released on the cytosolic side of the membrane and imported by a peroxisomal carrier. 4. ATP hydrolysis at the consensus nucleotide-binding site of CTS (smooth outline; see also Fig. 3) weakens contacts which maintain the NBD sandwich dimer. 5. NBD opening, to return to resting conformation. In symmetrical ABCDs such as ALDP, it is possible that two ATP molecules are hydrolysed in the transport cycle

3.2 Substrate Specificity and Thioester Cleavage

Whilst there is abundant evidence that CTS and other peroxisomal ABC transporters import substrates for β-oxidation, it has not been clear whether the transporters bind and translocate free acids or CoA esters. Activation by thioesterification to CoA is required for substrates to enter the β-oxidation pathway but plants encode a large family of acyl CoA synthetases (also known as acyl activating enzymes) with different subcellular localisations (Shockey and Browse 2011), therefore in theory, substrates could be activated either extra- or intraperoxisomally. Genetic deletion of the two peroxisomal long chain acyl-CoA synthetases (LACS6 and 7) causes a defect in seedling establishment in Arabidopsis, a phenotype also observed when the peroxisomal adenine nucleotide translocators that provide ATP for the activation reaction are down-regulated by RNAi (Fulda et al. 2004; Linka et al. 2008). Moreover, peroxisomal acetyl CoA synthetase mutants and *cts* alleles have a similar fluoroacetate resistance phenotype (Turner et al. 2005; Hooks et al. 2007). This suggests that free acids are translocated by CTS. However, *cts* mutants accumulate very long-chain acyl-CoAs and yeast peroxisomes containing the recombinant plant transporter exhibit ATPase activity which is stimulated by CoAs but not by free fatty acids (Footitt et al. 2002; Nyathi et al. 2010). Whilst this only constitutes indirect evidence for acyl-CoAs as substrates, supply of different substrates to yeast cells in which the plasma membrane had been selectively permeabilised indicated that only CoAs and not free fatty acids could support peroxisomal β-oxidation (Verleur et al. 1997).

One model which reconciles all available data is a scenario where the transporter accepts acyl-CoAs which are cleaved either before or during transport across the peroxisome membrane. Free acids would then be re-esterified inside the peroxisome, enabling β-oxidation (Fulda et al. 2004). Evidence to support this hypothesis has been obtained in yeast where ^{18}O labelling revealed that CoA is released during peroxisomal import of fatty acids (van Roermund et al. 2012). Whilst CTS and ALDP both complemented the yeast *pxa1/2Δ* mutant for fatty acid β-oxidation, neither was able to complement the *pxa1/2/faa2Δ* triple mutant which also lacks the peroxisomal acyl CoA synthetase, Faa2p (van Roermund et al. 2012; De Marcos Lousa et al. 2013), implying that peroxisomal activation is essential. Co-expression of CTS with either of the Arabidopsis peroxisomal LACS enzymes restored oleate β-oxidation, but not if LACS was mistargeted to the cytosol. Furthermore, detection of a subpopulation of LACS6/7 in a high molecular weight complex with CTS established a physical link between the transporter and peroxisomal acyl CoA synthetases (De Marcos Lousa et al. 2013). Remarkably, membrane preparations from insect cells expressing CTS exhibited an ATP-dependent acyl-CoA thioesterase activity which was markedly inhibited in the S810N mutant. These findings were presented as evidence for a model in which CTS itself cleaves the CoA moiety from acyl-CoAs, free fatty acids are translocated by the transporter and then re-esterified on the lumenal side of the membrane by closely associated acyl-CoA synthetases (De Marcos Lousa et al. 2013; Fig. 4).

The necessity for intraperoxisomal acyl CoA synthetase has been questioned for human cells, since β-oxidation of VLCFA-CoA by peroxisomes isolated from fibroblasts required the addition of NAD^+ but not CoA (Wiesinger et al. 2013). However, this conclusion is inconsistent with the requirement for Faa2p for VLCFA β-oxidation when ALDP is expressed in yeast (van Roermund et al. 2012) and the experiments with isolated human peroxisomes do not discount the possibility that CoA could be released on the lumenal side of the membrane by thioesterase activity of ALDP. Interestingly Faa2p is dispensable for VLCFA β-oxidation in yeast, since a population of the VLACS, Fat1p associates with the peroxisome membrane and is stabilised by Pxa1p/2p. Evidently ALDP and CTS are unable to interact productively with Fat1p and thus require the activity of Faa2p (van Roermund et al. 2012; De Marcos Lousa et al. 2013). Unequivocal proof that peroxisomal ABC transporters have an intrinsic thioesterase activity or whether a second protein fulfils this function awaits analysis of the purified, possibly reconstituted transporters, but intrinsic thioesterase/re-esterification is currently the model that best fits the available evidence.

3.3 Interacting Proteins and Transport Metabolons

In addition to lumenal enzymes, CTS also interacts with proteins at the cytosolic face of the peroxisome. Yeast two-hybrid screens using the Arabidopsis α/β hydrolase protein, CGI-58 as bait identified an interaction with NBD2 of CTS which was verified by pull-down assays (Park et al. 2013). In mammals, CGI-58 is a lipid droplet-associated protein that promotes the hydrolysis of TAG by activating adipose triglyceride lipase, but plants lack proteins that are important for this activity (Cantley et al. 2013). Although recombinant Arabidopsis CGI-58 exhibits TAG lipase activity in vitro, *cgi-58* mutants do not exhibit the seedling phenotypes associated with impaired mobilisation of storage lipids, nor do they have a defect in germination (James et al. 2010). However, *cgi-58* mutants accumulate oil bodies in leaves, exhibit 2,4-DB and IBA resistance and are impaired in jasmonate synthesis, implying that the wild-type protein has a function in β-oxidation in non-seed tissues. Thus it has been proposed that CGI-58 stimulates the activity of CTS, which is intriguing, given that it binds near the Walker B motif of NBD2 (Park et al. 2013), an interaction which might be predicted to impair formation of the NBD sandwich dimer. The precise role of CGI-58 remains to be determined with regard to CTS function, though the transport mechanism proposed above (De Marcos Lousa et al. 2013) raises the question of whether it might stimulate thioesterase activity or act as a thioesterase itself. The disparity between leaf and seedling phenotypes of *cgi-58* mutants also requires investigation, given that there is no obvious CGI-58 homologue in Arabidopsis which might contribute a redundant activity in seeds.

Regardless of whether the ABC transporter or an interacting thioesterase catalyses CoA cleavage, the translocation mechanism has interesting implications for

control of metabolism. Firstly, there are several examples of transporters which interact functionally with enzymes to create membrane transport metabolons involved in substrate channelling and metabolic regulation (Moraes and Reithmeier 2012). Since CTS can be considered to be a gateway for entry of diverse substrates into β-oxidation, the cleavage and re-esterification of transported molecules could allow the channelling or prioritising of substrates for metabolism dependent on the induction, activation or possibly specific recruitment of distinct acyl-CoA synthetases into a transport complex. In this context, it is significant that plant genomes encode multiple peroxisomal acyl activating enzymes (Shockey and Browse 2011; Hooks et al. 2012). Secondly, although it remains to be determined whether CoA is released in the cytosol or in the peroxisome matrix, the cleavage of CoA during transport is a potentially important consideration for the peroxisomal CoA budget since availability of this cofactor is thought to play a key role in regulating flux through β-oxidation (Hunt and Alexson 2002).

In mammals, the transport metabolon appears to be even more extensive since a search for interacting partners of ALDP and PMP70 provided evidence for a complex at the cytosolic face of the peroxisome consisting of enzymes with functions in fatty acid synthesis and activation (Hillebrand et al. 2012). The idea of a peroxisome transport metabolon is also consistent with earlier literature which reports the organisation of matrix enzymes into multienzyme complexes that allow efficient metabolic channelling with high flux rates (Reumann 2000). Thus it is conceivable that intimate protein–protein interactions control the regulation of metabolism from fatty acid synthesis, to peroxisomal import and degradation.

3.4 Other Regulatory Mechanisms

Examination of public array data reveals that *CTS* transcripts do not vary greatly in abundance during development or in response to various stimuli (Zimmermann et al. 2004), although protein levels do not always mirror transcript abundance. For example, seeds which have been prevented from germinating by exogenous application of abscisic acid (ABA) contain *CTS* transcripts but do not express detectable levels of CTS protein (Footitt et al. 2002), indicating that post-transcriptional regulation is important during germination and seedling establishment.

Possible post-translational regulatory mechanisms include phosphorylation: CTS phosphopeptides have been identified in a membrane protein phosphoproteomics study, but the functional significance has not been investigated (Engelsberger and Schulze 2012). Both ALDP and PMP70 are phosphorylated in vivo (Kemp et al. 2011) but it should be noted that regulation by phosphorylation could be quite different in ABCD transporters from different kingdoms. The activity of ABC transporters is also known to be modulated by the lipid environment and several (including human ABCD1, ABCC1 and ABCG2) have been detected in lipid rafts (Troost et al. 2004; Storch et al. 2007; Marbeuf-Gueye et al. 2007; Telbisz et al. 2007; Pollock et al. 2014). ALDP and PMP70 are

associated with distinct classes of lipid rafts in mammalian peroxisomes (Woudenberg et al. 2010) and it will be interesting to determine whether rafts play a role in modulating intrinsic transport activity and/or in the formation or stabilisation of protein complexes in plants.

4 Targeting

Relatively little is understood regarding targeting and insertion of peroxisomal membrane proteins (PMPs). PMPs, unlike other membrane proteins, are considered to be translated on free ribosomes and imported post-translationally into peroxisomes (Fujiki et al. 1984). Genetic and biochemical studies have shown that PMP targeting requires the multifunctional soluble, farnesylated protein, PEX19, which is thought to act as a chaperone/cycling receptor for PMPs, delivering them to the docking receptor, PEX3 at the peroxisome membrane (reviewed in Ma et al. 2011; Theodoulou et al. 2013). However, an alternative model has been proposed in which some, if not all PMPs are trafficked through the ER and delivered to peroxisomes in post-ER vesicles (van der Zand et al. 2010, 2012). In this model, export from the ER is dependent on PEX19 and other cytosolic factors. Although there is very strong evidence that some PMPs such as PEX3 and ascorbate peroxidase are trafficked through the ER, this is lacking for many integral membrane proteins (including ABCD proteins). Also, since peroxisome biogenesis occurs both by fission and also de novo from the ER, depending on the organism, tissue and metabolic state (Motley and Hettema 2007; Nuttall et al. 2011), PMPs may be trafficked via different routes in different situations.

Cross-kingdom targeting and complementation experiments show that trafficking of ABCD proteins is largely conserved between plants, animals and fungi, as peroxisomal ABCD transporters generally target correctly in heterologous hosts. However, some differences in specificity or components exist, since Arabidopsis PEX19 forms do not complement the yeast *pex19Δ* mutant (Yernaux et al. 2006; Saveria et al. 2007; Zhang et al. 2011; Nyathi et al. 2010, 2012). PMP19-binding sites and putative targeting signals (mPTS) have been identified empirically for ALDP and PMP70 and algorithms for prediction of PEX19 binding sites have been developed (Rottensteiner et al. 2004; Halbach et al. 2005; Kashiwayama et al. 2005, 2007; Iwashita et al. 2010; Schlüter et al. 2010). However, these tools are hampered by the fact that there is no simple amino acid sequence requirement for PEX19 binding and there may be multiple, internal mPTS and PEX19 binding sites within a given PMP. CTS is predicted to contain PEX19 sites in the N- and C-terminal halves of the protein. Accordingly, when expressed as artificial half-molecules, the N- and C-terminal halves each bind PEX19 and are targeted to the peroxisome in yeast (Nyathi et al. 2012). The presence of targeting information in both halves of the protein may result from the ancestral fusion event (as discussed above), but the retention of functionality argues for the importance of interactions between Pex19 with full-length CTS at more than one site. This may serve to maintain solubility

prior to insertion in the peroxisomal membrane, since large, polytopic membrane proteins are difficult for the cell to fold. Indeed misfolded peroxisomal ABC transporters are subject to quality control, as revealed by missense mutations associated with X-ALD, the majority of which result in markedly reduced protein levels at the peroxisome membrane (Zhang et al. 2011). The precise quality control mechanisms remain to be investigated but the existence of several checkpoints has been suggested (Takahashi et al. 2007). Interestingly, X-ALD mutants which were subject to proteasomal degradation in human fibroblasts were correctly targeted and stable when expressed in plant cells, an effect which was not wholly explicable by low temperature rescue of protein folding (Zhang et al. 2011).

5 Conclusions and Future Prospects

The last decade has seen tremendous advances in understanding peroxisomal ABC transporters but many questions remain. Studies using purified, reconstituted protein are urgently needed both to provide insight into the mechanisms and specificity of transport and to test the thioesterase activity and its relationship to ATP binding and hydrolysis. It would also be interesting to test the specificity of peroxisomal ABC transporters in different members of the green plant lineage and to relate this to different peroxisome functions in these organisms. Such investigations may afford valuable information regarding the molecular basis of substrate specificity and would underpin potential synthetic biology applications. Finally, elucidating the structure of peroxisomal ABC transporters remains a tantalising but extremely challenging goal.

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References

Antonenkov VD, Hiltunen JK (2012) Transfer of metabolites across the peroxisomal membrane. Biochim Biophys Acta 1822:1374–1386

Arai Y, Hayashi M, Nishimura M (2008) Proteomic analysis of highly purified peroxisomes from etiolated soybean cotyledons. Plant Cell Physiol 49:526–539

Bednarek P, Pislewska-Bednarek M, Svatos A, Schneider B, Doubsky J, Mansurova M, Humphry M, Consonni C, Panstruga R, Sanchez-Vallet A, Molina A, Schulze-Lefert P (2009) A glucosinolate metabolism pathway in living plant cells mediates broad-spectrum antifungal defense. Science 323:101–106

Boisnard S, Espagne E, Zickler D, Bourdais A, Riquet AL, Berteaux-Lecellier V (2009) Peroxisomal ABC transporters and beta-oxidation during the life cycle of the filamentous fungus Podospora anserina. Fungal Genet Biol 46:55–66

Buchanan-Wollaston V, Page T, Harrison E, Breeze E, Lim PO, Nam HG, Lin JF, Wu SH, Swidzinski J, Ishizaki K, Leaver CJ (2005) Comparative transcriptome analysis reveals

significant differences in gene expression and signalling pathways between developmental and dark/starvation-induced senescence in Arabidopsis. Plant J 42:567–585

Bussell JD, Reichelt M, Wiszniewski AA, Gershenzon J, Smith SM (2013) Peroxisomal ABC transporter CTS and the multifunctional protein AIM1 are required for production of benzoylated metabolites in Arabidopsis seeds. Plant Physiol Nov 19. [Epub ahead of print]

Bussell JD, Reichelt M, Wiszniewski AA, Gershenzon J, Smith SM (2014) Peroxisomal ABC transporter CTS and the multifunctional protein AIM1 are required for production of benzoylated metabolites in Arabidopsis seeds. Plant Physiol 164:48–54

Cantley JL, Yoshimura T, Camporez JP, Zhang D, Jornayvaz FR, Kumashiro N, Guebre-Egziabher F, Jurczak MJ, Kahn M, Guigni BA, Serr J, Hankin J, Murphy RC, Cline GW, Bhanot S, Manchem VP, Brown JM, Samuel VT, Shulman GI (2013) CGI-58 knockdown sequesters diacylglycerols in lipid droplets/ER-preventing diacylglycerol-mediated hepatic insulin resistance. Proc Natl Acad Sci U S A 110:1869–1874

Carrera E, Holman T, Medhurst A, Peer W, Schmuths H, Footitt S, Theodoulou FL, Holdsworth MJ (2007) Gene expression profiling reveals defined functions of the ABC transporter *COMATOSE* late in phase II of germination. Plant Physiol 143:1669–1679

Clay NK, Adio AM, Denoux C, Jander G, Ausubel FM (2009) Glucosinolate metabolites required for an Arabidopsis innate immune response. Science 323:95–101

Coelho D, Kim JC, Miousse IR, Fung S, du Moulin M, Buers I, Suormala T, Burda P, Frapolli M, Stucki M, Nürnberg P, Thiele H, Robenek H, Höhne W, Longo N, Pasquali M, Mengel E, Watkins D, Shoubridge EA, Majewski J, Rosenblatt DS, Fowler B, Rutsch F, Baumgartner MR (2012) Mutations in ABCD4 cause a new inborn error of vitamin B12 metabolism. Nat Genet 44:1152–1155

Croft MT, Lawrence AD, Raux-Deery E, Warren MJ, Smith AG (2005) Algae acquire vitamin B12 through a symbiotic relationship with bacteria. Nature 438:90–93

Dave A, Hernández ML, He Z, Andriotis VM, Vaistij FE, Larson TR, Graham IA (2011) 12-oxo-phytodienoic acid accumulation during seed development represses seed germination in Arabidopsis. Plant Cell 23:583–599

Dawson RJ, Locher KP (2006) Structure of a bacterial multidrug ABC transporter. Nature 443: 180–185

De Marcos Lousa C, van Roermund CW, Postis VLG, Dietrich D, Kerr ID, Wanders RJA, Baldwin SA, Baker A, Theodoulou FL (2013) Intrinsic acyl-CoA thioesterase activity of a peroxisomal ABC transporter is required for transport and metabolism of fatty acids. Proc Natl Acad Sci U S A 110:1279–1284

Dietrich D, Schmuths H, De Marcos LC, Baldwin JM, Baldwin SA, Baker A, Theodoulou FL, Holdsworth MJ (2009) Mutations in the Arabidopsis peroxisomal ABC transporter COMATOSE allow differentiation between multiple functions *in planta*: insights from an allelic series. Mol Biol Cell 20:530–543

Engelsberger WR, Schulze WX (2012) Nitrate and ammonium lead to distinct global dynamic phosphorylation patterns when resupplied to nitrogen-starved Arabidopsis seedlings. Plant J 69:978–995

Eubel H, Meyer EH, Taylor NL, Bussell JD, O'Toole N, Heazlewood JL, Castleden I, Small ID, Smith SM, Millar AH (2008) Novel proteins, putative membrane transporters, and an integrated metabolic network are revealed by quantitative proteomic analysis of Arabidopsis cell culture peroxisomes. Plant Physiol 148:1809–1829

Fan J, Quan S, Orth T, Awai C, Chory J, Hu J (2005) The Arabidopsis PEX12 gene is required for peroxisome biogenesis and is essential for development. Plant Physiol 139:231–239

Footitt S, Slocombe SP, Larner V, Kurup S, Wu Y, Larson T, Graham I, Baker A, Holdsworth M (2002) Control of germination and lipid mobilization by COMATOSE, the Arabidopsis homologue of human ALDP. EMBO J 21:2912–2922

Footitt S, Marquez J, Schmuths H, Baker A, Theodoulou FL, Holdsworth M (2006) Analysis of the role of *COMATOSE* and peroxisomal beta-oxidation in the determination of germination potential in *Arabidopsis*. J Exp Bot 57:2805–2814

Footitt S, Dietrich D, Fait A, Fernie A, Holdsworth MJ, Baker A, Theodoulou FL (2007) The COMATOSE ABC transporter is required for full fertility in Arabidopsis. Plant Physiol 144: 1467–1480

Fujiki Y, Rachubinski RA, Lazarow PB (1984) Synthesis of a major integral membrane polypeptide of rat liver peroxisomes on free polysomes. Proc Natl Acad Sci U S A 81:7127–7131

Fulda M, Schnurr J, Abbadi A, Heinz E, Browse J (2004) Peroxisomal Acyl-CoA synthetase activity is essential for seedling development in Arabidopsis thaliana. Plant Cell 16:394–405

Genin EC, Geillon F, Gondcaille C, Athias A, Gambert P, Trompier D, Savary S (2011) Substrate specificity overlap and interaction between adrenoleukodystrophy protein (ALDP/ABCD1) and adrenoleukodystrophy-related protein (ALDRP/ABCD2). J Biol Chem 286:8075–8084

Girzalsky W, Saffian D, Erdmann R (2010) Peroxisomal protein translocation. Biochim Biophys Acta 1803:724–731

Guimarães CP, Domingues P, Aubourg P, Fouquet F, Pujol A, Jimenez-Sanchez G, Sá-Miranda C, Azevedo JE (2004) Mouse liver PMP70 and ALDP: homomeric interactions prevail in vivo. Biochim Biophys Acta 1689:235–243

Halbach A, Lorenzen S, Landgraf C, Volkmer-Engert R, Erdmann R, Rottensteiner H (2005) Function of the PEX19-binding site of human adrenoleukodystrophy protein as targeting motif in man and yeast. PMP targeting is evolutionarily conserved. J Biol Chem 280:21176–21182

Hayashi M, Nito K, Takei-Hoshi R, Yagi M, Kondo M, Suenaga A, Yamaya T, Nishimura M (2002) Ped3p is a peroxisomal ATP-binding cassette transporter that might supply substrates for fatty acid beta-oxidation. Plant Cell Physiol 43:1–11

Hettema EH, van Roermund CW, Distel B, van den Berg M, Vilela C, Rodrigues-Pousada C, Wanders RJ, Tabak HF (1996) The ABC transporter proteins Pat1 and Pat2 are required for import of long-chain fatty acids into peroxisomes of Saccharomyces cerevisiae. EMBO J 15: 3813–3822

Hillebrand M, Gersting SW, Lotz-Havla AS, Schäfer A, Rosewich H, Valerius O, Muntau AC, Gärtner J (2012) Identification of a new fatty acid synthesis-transport machinery at the peroxisomal membrane. J Biol Chem 287:210–221

Hooks MA, Turner JE, Murphy EC, Johnston KA, Burr S, Jarosławski S (2007) The Arabidopsis ALDP protein homologue COMATOSE is instrumental in peroxisomal acetate metabolism. Biochem J 406:399–406

Hooks KB, Turner JE, Graham IA, Runions J, Hooks MA (2012) GFP-tagging of Arabidopsis acyl-activating enzymes raises the issue of peroxisome-chloroplast import competition versus dual localization. J Plant Physiol 169:1631–1638

Hu J, Baker A, Bartel B, Linka N, Mullen RT, Reumann S, Zolman BK (2012) Plant peroxisomes: biogenesis and function. Plant Cell 24:2279–2303

Hunt MC, Alexson SE (2002) The role acyl-CoA thioesterases play in mediating intracellular lipid metabolism. Prog Lipid Res 41:99–130

Iwashita S, Tsuchida M, Tsukuda M, Yamashita Y, Emi Y, Kida Y, Komori M, Kashiwayama Y, Imanaka T, Sakaguchi M (2010) Multiple organelle-targeting signals in the N-terminal portion of peroxisomal membrane protein PMP70. J Biochem 147:581–590

James CN, Horn PJ, Case CR, Gidda SK, Zhang D, Mullen RT, Dyer JM, Anderson RG, Chapman KD (2010) Disruption of the Arabidopsis CGI-58 homologue produces Chanarin-Dorfman-like lipid droplet accumulation in plants. Proc Natl Acad Sci U S A 107:17833–17838

Kanai M, Nishimura M, Hayashi M (2010) A peroxisomal ABC transporter promotes seed germination by inducing pectin degradation under the control of ABI5. Plant J 62:936–947

Kashiwayama Y, Asahina K, Shibata H, Morita M, Muntau AC, Roscher AA, Wanders RJ, Shimozawa N, Sakaguchi M, Kato H, Imanaka T (2005) Role of Pex19p in the targeting of PMP70 to peroxisome. Biochim Biophys Acta 1746:116–128

Kashiwayama Y, Asahina K, Morita M, Imanaka T (2007) Hydrophobic regions adjacent to transmembrane domains 1 and 5 are important for the targeting of the 70-kDa peroxisomal membrane protein. J Biol Chem 282:33831–33844

Kashiwayama Y, Seki M, Yasui A, Murasaki Y, Morita M, Yamashita Y, Sakaguchi M, Tanaka Y, Imanaka T (2009) 70-kDa peroxisomal membrane protein related protein (P70R/ABCD4) localizes to endoplasmic reticulum not peroxisomes, and NH2-terminal hydrophobic property determines the subcellular localization of ABC subfamily D proteins. Exp Cell Res 315: 190–205

Kelly AA, Quettier AL, Shaw E, Eastmond PJ (2011) Seed storage oil mobilization is important but not essential for germination or seedling establishment in Arabidopsis. Plant Physiol 157: 866–875

Kemp S, Theodoulou FL, Wanders RJA (2011) Mammalian peroxisomal ABC transporters: from endogenous substrates to pathology and clinical significance. Br J Pharmacol 164:1753–1766

Kemp S, Berger J, Aubourg P (2012) X-linked adrenoleukodystrophy: clinical, metabolic, genetic and pathophysiological aspects. Biochim Biophys Acta 1822:1465–1474

Kunz HH, Scharnewski M, Feussner K, Feussner I, Flügge UI, Fulda M, Gierth M (2009a) The ABC transporter PXA1 and peroxisomal beta-oxidation are vital for metabolism in mature leaves of Arabidopsis during extended darkness. Plant Cell 21:2733–2749

Kunz HH, Scharnewski M, von Berlepsch S, Shahi S, Fulda M, Flügge UI, Gierth M (2009b) Nocturnal energy demand in plants: insights from studying mutants impaired in β-oxidation. Plant Signal Behav 5:842–844

Lazo O, Contreras M, Hashmi M, Stanley W, Irazu C, Singh I (1988) Peroxisomal lignoceroyl-CoA ligase deficiency in childhood adrenoleukodystrophy and adrenomyeloneuropathy. Proc Natl Acad Sci U S A 85:7647–7651

Linka N, Theodoulou FL (2013) Metabolite transporters of the plant peroxisomal membrane: known and unknown. Subcell Biochem 69:169–194

Linka N, Theodoulou FL, Haslam RP, Napier JA, Linka M, Neuhaus HE, Weber APM (2008) Peroxisomal ATP import is essential for seedling development in *Arabidopsis thaliana*. Plant Cell 20:3241–3257

Lipka V, Dittgen J, Bednarek P, Bhat R, Wiermer M, Stein M, Landtag J, Brandt W, Rosahl S, Scheel D, Llorente F, Molina A, Parker J, Somerville S, Schulze-Lefert P (2005) Pre- and postinvasion defenses both contribute to nonhost resistance in Arabidopsis. Science 310: 1180–1183

Ma C, Agrawal G, Subramani S (2011) Peroxisome assembly: matrix and membrane protein biogeneis. J Cell Biol 193:7–16

Marbeuf-Gueye C, Stierle V, Sudwan P, Salerno M, Garnier-Suillerot A (2007) Perturbation of membrane microdomains in GLC4 multidrug-resistant lung cancer cells- modification of ABCC1 (MRP1) localization and functionality. FEBS J 274:1470–1480

Mendiondo GM, Medhurst A, van Roermund CW, Zhang A, Devonshire J, Scholefield D, Fernández J, Axcell B, Ramsay L, Waterham HR, Waugh R, Theodoulou FL, Holdsworth MJ (2014) Barley has two peroxisomal ABC transporters with multiple functions in β-oxidation. J Exp Bot (in press)

Moody JE, Millen L, Binns D, Hunt JF, Thomas PJ (2002) Cooperative, ATP-dependent association of nucleotide binding cassettes during the catalytic cycle of ATP-binding cassette transporters. J Biol Chem 277:21111–21114

Moraes TF, Reithmeier RAF (2012) Membrane transport metabolons. Biochim Biophs Acta 1818: 2687–2706

Morita M, Imanaka T (2012) Peroxisomal ABC transporters: structure, function and role in disease. Biochim Biophys Acta 1822:1387–1396

Mosser J, Douar AM, Sarde CO, Kioschis P, Feil R, Moser H, Poustka AM, Mandel JL, Aubourg P (1993) Putative X-linked adrenoleukodystrophy gene shares unexpected homology with ABC transporters. Nature 361:726–730

Motley AM, Hettema EH (2007) Yeast peroxisomes multiply by growth and division. J Cell Biol 178:399–410

Nito K, Kamigaki A, Kondo M, Hayashi M, Nishimura M (2007) Functional classification of Arabidopsis peroxisome biogenesis factors proposed from analyses of knockdown mutants. Plant Cell Physiol 48:763–74

Nuttall JM, Motley A, Hettema EH (2011) Peroxisome biogenesis: recent advances. Curr Opin Cell Biol 23:421–426

Nyathi Y, De Marcos LC, van Roermund CW, Wanders RJA, Baldwin SA, Theodoulou FL, Baker A (2010) The Arabidopsis peroxisomal ABC transporter, COMATOSE, complements the *Saccharomyces cerevisiae pxa1 pxa2Δ* double mutant for metabolism of long chain fatty acids and exhibits fatty acyl-CoA stimulated ATPase activity. J Biol Chem 285:29892–29902

Nyathi N, Zhang X, Baldwin J, Bernhardt K, Johnson B, Baldwin SA, Theodoulou FL, Baker A (2012) Pseudo half-molecules of the ABC transporter, COMATOSE, bind Pex19 and target to peroxisomes independently but are both required for activity. FEBS Lett 586:2280–2286

Orsi CH, Tanksley SD (2009) Natural variation in an ABC transporter gene associated with seed size evolution in tomato species. PLoS Genet 5:e1000347

Park S, Gidda SK, James CN, Horn PJ, Khuu N, Seay DC, Keereetaweep J, Chapman KD, Mullen RT, Dyer JM (2013) The α/β hydrolase CGI-58 and peroxisomal transport protein PXA1 coregulate lipid homeostasis and signaling in Arabidopsis. Plant Cell 25:1726–1739

Pinfield-Wells H, Rylott EL, Gilday AD, Graham S, Job K, Larson TR, Graham IA (2005) Sucrose rescues seedling establishment but not germination of Arabidopsis mutants disrupted in peroxisomal fatty acid catabolism. Plant J 43:861–872

Pollock NL, McDevitt CA, Collins R, Niesten PH, Prince S, Kerr ID, Ford RC, Callaghan R (2014) Improving the stability and function of purified ABCB1 and ABCA4: The influence of membrane lipids. Biochim Biophys Acta 1838:134–147

Procko E, O'Mara ML, Bennett WF, Tieleman DP, Gaudet R (2009) The mechanism of ABC transporters: general lessons from structural and functional studies of an antigenic peptide transporter. FASEB J 23:1287–1302

Qualley AV, Widhalm JR, Adebesin F, Kish CM, Dudareva N (2012) Completion of the core β-oxidative pathway of benzoic acid biosynthesis in plants. Proc Natl Acad Sci U S A 109: 16383–16388

Reumann S (2000) The structural properties of plant peroxisomes and their metabolic significance. Biol Chem 381:639–648

Reumann S, Babujee L, Ma C, Wienkoop S, Siemsen T, Antonicelli GE, Rasche N, Lüder F, Weckwerth W, Jahn O (2007) Proteome analysis of Arabidopsis leaf peroxisomes reveals novel targeting peptides, metabolic pathways, and defense mechanisms. Plant Cell 19: 3170–3193

Reumann S, Quan S, Aung K, Yang P, Manandhar-Shrestha K, Holbrook D, Linka N, Switzenberg R, Wilkerson CG, Weber AP, Olsen LJ, Hu J (2009) In-depth proteome analysis of Arabidopsis leaf peroxisomes combined with in vivo subcellular targeting verification indicates novel metabolic and regulatory functions of peroxisomes. Plant Physiol 150:125–143

Rottensteiner H, Kramer A, Lorenzen S, Stein K, Landgraf C, Volkmer-Engert R, Erdmann R (2004) Peroxisomal membrane proteins contain common Pex19p-binding sites that are an integral part of their targeting signals. Mol Biol Cell 15:3406–3417

Russell L (1998) Isolation and analysis of mutants in *Arabidopsis thaliana* disrupted in the transition between dormancy and germination. PhD thesis, University of Bristol

Russell L, Larner V, Kurup S, Bougourd S, Holdsworth M (2000) The Arabidopsis COMATOSE locus regulates germination potential. Development 127:3759–3767

Saveria T, Halbach A, Erdmann R, Volkmer-Engert R, Landgraf C, Rottensteiner H, Parsons M (2007) Conservation of PEX19-binding motifs required for protein targeting to mammalian peroxisomal and trypanosome glycosomal membranes. Eukaryot Cell 6:1439–1449

Schlüter A, Real-Chicharro A, Gabaldón T, Sánchez-Jiménez F, Pujol A (2010) PeroxisomeDB 2.0: an integrative view of the global peroxisomal metabolome. Nucleic Acids Res 38:D800–5

Shani N, Valle D (1996) A Saccharomyces cerevisiae homolog of the human adrenoleukodystrophy transporter is a heterodimer of two half ATP-binding cassette transporters. Proc Natl Acad Sci U S A 93:11901–11906

Shockey J, Browse J (2011) Genome-level and biochemical diversity of the acyl-activating enzyme superfamily in plants. Plant J 66:143–160

Slocombe SP, Cornah J, Pinfield-Wells H, Soady K, Zhang Q, Gilday A, Dyer JM, Graham IA (2009) Oil accumulation in leaves directed by modification of fatty acid breakdown and lipid synthesis pathways. Plant Biotechnol J 7:694–703

Smith PC, Karpowich N, Millen L, Moody JE, Rosen J, Thomas PJ, Hunt JF (2002) ATP binding to the motor domain of an ABC transporter drives formation of a nucleotide sandwich dimer. Mol Cell 10:139–149

Sparkes IA, Brandizzi F, Slocombe SP, El-Shami M, Hawes C, Baker A (2003) An Arabidopsis pex10 null mutant is embryo lethal, implicating peroxisomes in an essential role during plant embryogenesis. Plant Physiol 133:1809–1819

Storch CH, Ehehalt R, Haefeli WE, Weiss J (2007) Localization of the human breast cancer resistance protein (BCRP/ABCG2) in lipid rafts/caveolae and modulation of its activity by cholesterol in vitro. J Pharmacol Exp Ther 323:257–264

Strader LC, Culler AH, Cohen JD, Bartel B (2010) Conversion of endogenous indole-3-butyric acid to indole-3-acetic acid drives cell expansion in Arabidopsis seedlings. Plant Physiol 153: 1577–1586

Strader LC, Wheeler DL, Christensen SE, Berens JC, Cohen JD, Rampey RA, Bartel B (2011) Multiple facets of Arabidopsis seedling development require indole-3-butyric acid-derived auxin. Plant Cell 23:984–999

Takahashi N, Morita M, Maeda T, Harayama Y, Shimozawa N, Suzuki Y, Furuya H, Sato R, Kashiwayama Y, Imanaka T (2007) Adrenoleukodystrophy: subcellular localization and degradation of adrenoleukodystrophy protein (ALDP/ABCD1) with naturally occurring missense mutations. J Neurochem 101:1632–1643

Taler D, Galperin M, Benjamin I, Cohen Y, Kenigsbuch D (2004) Plant eR genes that encode photorespiratory enzymes confer resistance against disease. Plant Cell 16:172–84

Telbisz A, Müller M, Ozvegy-Laczka C, Homolya L, Szente L, Varadi A, Sarkadi B (2007) Membrane cholesterol selectively modulates the activity of the human ABCG2 multidrug transporter. Biochim Biophys Acta 1768:2698–2713

Theodoulou FL, Job K, Slocombe SP, Footitt S, Holdsworth M, Baker A, Larson TR, Graham IA (2005) Jasmonic acid levels are reduced in COMATOSE ABC transporter mutants: implications for transport of jasmonate precursors into peroxisomes. Plant Physiol 137:835–840

Theodoulou FL, Holdsworth M, Baker A (2006) Peroxisomal ABC transporters. FEBS Lett 580: 1139–1155

Theodoulou FL, Zhang X, De Marcos LC, Nyathi Y, Baker A (2011) Peroxisomal transport systems: roles in signaling and metabolism. In: Geisler M, Venema K (eds) Transporters and pumps in plant signaling, vol 7, Signaling and communication in plants. Springer, Berlin, Heidelberg, pp 327–351

Theodoulou FL, Bernhardt K, Linka N, Baker A (2013) Peroxisome membrane proteins: multiple trafficking routes and multiple functions? Biochem J 451:345–352

Thevenieau F, Le Dall MT, Nthangeni B, Mauersberger S, Marchal R, Nicaud JM (2007) Characterization of Yarrowia lipolytica mutants affected in hydrophobic substrate utilization. Fungal Genet Biol 44:531–542

Troost J, Lindenmaier H, Haefeli WE, Weiss J (2004) Modulation of cellular cholesterol alters P-glycoprotein activity in multidrug-resistant cells. Mol Pharmacol 66:1332–1339

Turner JE, Greville K, Murphy EC, Hooks MA (2005) Characterization of Arabidopsis fluoroacetate-resistant mutants reveals the principal mechanism of acetate activation for entry into the glyoxylate cycle. J Biol Chem 280:2780–2787

Tyra HM, Linka M, Weber AP, Bhattacharya D (2007) Host origin of plastid solute transporters in the first photosynthetic eukaryotes. Genome Biol 8:R212

van der Graaff E, Schwacke R, Schneider A, Desimone M, Flügge UI, Kunze R (2006) Transcription analysis of Arabidopsis membrane transporters and hormone pathways during developmental and induced leaf senescence. Plant Physiol 141:776–792

van der Zand A, Braakman I, Tabak HF (2010) Peroxisomal membrane proteins insert into the endoplasmic reticulum. Mol Biol Cell 21:2057–2065

van der Zand A, Gent J, Braakman I, Tabak HF (2012) Biochemically distinct vesicles from the endoplasmic reticulum fuse to form peroxisomes. Cell 149:397–409

van Roermund CW, Visser WF, Ijlst L, van Cruchten A, Boek M, Kulik W, Waterham HR, Wanders RJ (2008) The human peroxisomal ABC half transporter ALDP functions as a homodimer and accepts acyl-CoA esters. FASEB J 22:4201–4208

van Roermund CW, Visser WF, Ijlst L, Waterham HR, Wanders RJ (2011) Differential substrate specificities of human ABCD1 and ABCD2 in peroxisomal fatty acid β-oxidation. Biochim Biophys Acta 1811:148–152

van Roermund CW, Ijlst L, Majczak W, Waterham HR, Folkerts H, Wanders RJ, Hellingwerf KJ (2012) Peroxisomal fatty acid uptake mechanism in Saccharomyces cerevisiae. J Biol Chem 287:20144–20153

van Roermund CW, Ijlst L, Wagemans T, Wanders RJ, Waterham HR (2013) A role for the human peroxisomal half-transporter ABCD3 in the oxidation of dicarboxylic acids. Biochim Biophys Acta. doi:10.1016/j.bbalip.2013.12.001

Verleur N, Hettema EH, van Roermund CW, Tabak HF, Wanders RJ (1997) Transport of activated fatty acids by the peroxisomal ATP-binding-cassette transporter Pxa2 in a semi-intact yeast cell system. Eur J Biochem 249:657–661

Verrier PJ, Bird D, Burla B, Dassa E, Forestier C, Geisler M, Klein M, Kolukisaoglu Ü, Lee Y, Martinoia E, Murphy A, Rea PA, Samuels L, Schulz B, Spalding EP, Yazaki K, Theodoulou FL (2008) Plant ABC proteins- a unified nomenclature and updated inventory. Trends Plant Sci 13:151–159

Wanders RJ (2004) Peroxisomes, lipid metabolism, and peroxisomal disorders. Mol Genet Metab 83:16–27

Wanders RJ, van Roermund CW, Schutgens RB, van den Bosch H, Tager JM (1988a) Impaired ability of peroxisomes to activate very-long-chain fatty acids in X-linked adrenoleukodystrophy. Lancet 2:170

Wanders RJ, van Roermund CW, van Wijland MJ, Schutgens RB, van den Bosch H, Schram AW, Tager JM (1988b) Direct demonstration that the deficient oxidation of very long chain fatty acids in X-linked adrenoleukodystrophy is due to an impaired ability of peroxisomes to activate very long chain fatty acids. Biochem Biophys Res Commun 153:618–624

Wanders RJ, Visser WF, van Roermund CW, Kemp S, Waterham HR (2007) The peroxisomal ABC transporter family. Pflügers Arch 453:719–734

Wiesinger C, Kunze M, Regelsberger G, Forss-Petter S, Berger J (2013) Impaired very long-chain acyl-CoA β-oxidation in human X-linked adrenoleukodystrophy fibroblasts is a direct consequence of ABCD1 transporter dysfunction. J Biol Chem 288:19269–19279

Woudenberg J, Rembacz KP, Hoekstra M, Pellicoro A, van den Heuvel FA, Heegsma J, van Ijzendoorn SC, Holzinger A, Imanaka T, Moshage H, Faber KN (2010) Lipid rafts are essential for peroxisome biogenesis in HepG2 cells. Hepatology 52:623–633

Yang Z, Ohlrogge JB (2009) Turnover of fatty acids during natural senescence of Arabidopsis, Brachypodium, and switchgrass and in Arabidopsis beta-oxidation mutants. Plant Physiol 150: 1981–1989

Yernaux C, Fransen M, Brees C, Lorenzen S, Michels PA (2006) Trypanosoma brucei glycosomal ABC transporters: identification and membrane targeting. Mol Membr Biol 23:157–172

Zhang X, De Marcos LC, Schutte-Lensink N, Ofman R, Wanders RJ, Baldwin SA, Baker A, Kemp S, Theodoulou FL (2011) Conservation of targeting but divergence in function and quality control of peroxisomal ABC transporters: an analysis using cross-kingdom expression. Biochem J 436:547–557

Zimmermann P, Hirsch-Hoffmann M, Hennig L, Gruissem W (2004) GENEVESTIGATOR. Arabidopsis microarray database and analysis toolbox. Plant Physiol 136:2621–2632

Zolman BK, Silva ID, Bartel B (2001) The Arabidopsis pxa1 mutant is defective in an ATP-binding cassette transporter-like protein required for peroxisomal fatty acid beta-oxidation. Plant Physiol 127:1266–1278

Plastidic ABC Proteins

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Abstract ATP-binding cassette (ABC) proteins belong to a large family of proteins which mostly catalyze ATP-driven membrane translocation. They are found throughout the tree of life, from Archaea to Eukarya. In plants, they are known to perform essential functions in multiple subcellular compartments, though the locations of each ABC protein in a given species have not yet been defined. Here, we mine available proteomic, targeting, and fluorescence localization data to identify the subcellular location of each predicted ABC protein of Arabidopsis. Then we examine the functions of plastidic ABC proteins in detail. Transport functions are particularly important to the plastid, where a diverse group of substrates must be transported to allow specialized plastid metabolic functions to occur. Plastidic transporters that remain unidentified have been hypothesized based on known substrate translocation events. We attempt to narrow this gap by compiling available information about each plastidic ABC protein, including the primary literature, Chloroplast 2010 database information, transcriptomic data, coexpression analyses, and phylogenic data. This process ultimately allows us to make new hypotheses about the function of several plastidic ABC proteins and to identify a new family member of the AtABCI subfamily.

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1 An Introduction to ABC Proteins and Plastids

Cells require separation from the environment in order to maintain their many chemical reactions at non-equilibrium, which is a hallmark of life. This separation is achieved through hydrophobic, semipermeable membranes, which alone allow controlled connection of the inside of the cell and the environment. Complete disconnection is not desirable, as the cells need to interact with the environment to obtain necessary raw materials. Thus, there is a strong need for transporters in membranes that allow controlled interaction with the environment. Throughout the tree of life, Archaea, Bacteria, and Eukaryota, the ATP-binding cassette (ABC) transporter family is one of the most abundant transporter families (Higgins and Linton 2003). This is best exemplified in the *E. coli* genome, where genes encoding ABC transporters make up 5 % of all genes (Linton and Higgins 1998).

In most cases, ABC transporters couple membrane translocation of a diverse group of substrates to the energizing power of ATP-hydrolysis, allowing concentration of the substrate on one side of the cell membrane, or in many eukaryotes, the subcellular compartment membranes (Higgins 1992). These transporters have conserved, recognizable protein domains known as nucleotide-binding domains (NBDs) and transmembrane domains (TMDs). Two TMDs and two NBDs are the minimum domains required to make a functional ABC transporter (Hollenstein et al. 2007). There may or may not be a third domain associated, usually a separate polypeptide chain with a substrate-binding domain (van der Heide and Poolman 2002). Of these domains, the NBD is the most readily identifiable. It contains the Walker A and Walker B motifs, as well as an α-helical region with the ABC "signature motif" LSGGQ (Rees et al. 2009). The TMD also has recognizable consensus sequences, but they can be one of three distinct folds (Locher 2009). While the first two are undisputed as ABC transporters, the third, specific to cobalt, cobalamin, or heme transporters, has recently been reclassified as a separate family (Rodionov et al. 2009).

The ABC transporter superfamily has been divided into multiple subfamilies. Among prokaryotic ABC transporters, the direction of transport, import or export, also forms the basis of two distinct phylogenetic clades (Saurin et al. 1999). Prokaryotic ABC importer genes typically encode a single domain (i.e., one TMD, NBD, or substrate-binding domain). Proteins corresponding to each domain associate to form a transporter complex with functional stoichiometry: two NBDs and two TMDs, frequently with a substrate-binding protein either stably or transiently associated (van der Heide and Poolman 2002). In contrast, the large majority of prokaryotic ABC exporter genes encode two to four fused domains. When two domains are encoded, the protein is referred to as a "half transporter," as it typically includes a single TMD and NBD. In vivo, the resulting polypeptides homo- or heterodimerize to form a functional transporter. When four domains are encoded, two TMDs and two NBDs usually are present on the same polypeptide and form "full transporters" without dimerization. For further information about ABC

transporter architecture see reviews (Locher 2009; Hollenstein et al. 2007; Holland 2011).

In eukaryotes, the group of ABC transporters most similar in structure to prokaryotic exporters has expanded greatly, and it has been further divided into subfamilies A–H based on domain organization and sequence similarity (Bouige et al. 2002; Dean and Annilo 2005). These have been referred to as eukaryotic transporters, partially because studied vertebrate models do not have genes similar to prokaryotic ABC importers (Saurin et al. 1999). However, in algae and land plants, genes similar to both prokaryotic ABC exporters and prokaryotic ABC importers exist. Thus, when considering plant ABC transporters we refer to "fused ABC transporters," i.e., similar to prokaryotic ABC exporters and "multipartite ABC transporters," i.e., similar to prokaryotic ABC importers. To include the multipartite ABC transporters found in plants into the alphabetic ABC transporter subfamily naming system originally established for vertebrates, they have been given the subfamily designation I (Verrier et al. 2008).

In addition to the multiple classifications of ABC transporters, some proteins containing domains found in ABC transporters have diversified to have roles other than transport including transcriptional regulation and iron-sulfur cluster metabolism, possibly because of the ancient origins of the family. Although these proteins retain similarity to ABC transporter NBD domains at the amino acid level, they no longer associate with a functional transporter (Higgins 1992). Generally, these can be called ABC proteins, but not ABC transporters. For classification purposes, genes encoding single NBD domains have been included in subfamily I (Verrier et al. 2008), while those encoding fused NBD domains are in subfamily F (Bouige et al. 2002).

When studied in eukaryotes, it is particularly important to consider the function of ABC transporters in their biological context. Because their functions frequently allow sequestration of molecules within specific compartments, knowing their substrate is equally important to knowing what compartment they interact with. In plants, the plastid is a particularly interesting compartment, as it provides the plant cell with many necessary functions, from specialized metabolic processes like photosynthesis to storage and signaling roles. In order to perform these functions, a large variety of molecules must be transported through the plastid envelope membranes.

When considered in an evolutionary context, the plastid envelope transporters are of even higher interest. The plastid arose from an event or events which allowed an ancestral cyanobacterial endosymbiont to be engulfed and then incorporated into a eukaryotic host cell (Keeling 2010). In the new plastid, the original cyanobacterial transporters would have the wrong orientation or be transporting the wrong substrates for the needs of the new cell. Accordingly, a reorganization of available transporters from both the eukaryotic host and the endosymbiont was a likely prerequisite for successful integration (Weber and Linka 2011). During this process, the plastid acquired a number of transporters characteristic of eukaryotes, including fused ABC transporters. At the same time, it retained many of its multipartite ABC transporters of prokaryotic origin, making the plastid and

plastid-carrying organisms, unique among eukaryotes for having both types of transporters.

Currently, more is known about plastid metabolism than plastid transporters. Multiple transporter functions beyond the currently characterized ones have been inferred to be necessary for normal plastid functions within land plants (Rolland et al. 2012). It is likely that some of the inferred transporter functions are performed by ABC transporters, as few plastidic ABC transporters have been studied in detail.

Thus, it is the aim of this chapter to analyze the subcellular locations of plant ABC proteins and to provide an indepth analysis of each one found in the plastid. These analyses not only cover available data from primary publications but also consolidate relevant information from publically available datasets to give the best possible description of each plastidic ABC protein.

2 Subcellular Locations of Plant ABC Proteins

Although the phylogenies of ABC proteins have been thoroughly characterized in plants (Verrier et al. 2008; Kang et al. 2011; Pang et al. 2013), their locations are less clear. As with many types of plant proteins, they are either derived from the eukaryotic host or the ancient ancestors of mitochondria and plastids. However, protein subcellular locations cannot be determined by lineage alone as plastids and mitochondria of modern plants contain proteins of both prokaryotic and eukaryotic origin (Reyes-Prieto et al. 2007; Gray 2012). In theory, targeting information needed to target modern proteins to their organellar destination should allow prediction of those proteins. However in reality, the best targeting programs range from 70 to 80 % accuracy (Kaundal et al. 2010). In recent years, proteomics studies have done much to define subcellular locations using high-throughput data, but these too fall short of finding all proteins (van Wijk and Baginsky 2011). For these reasons, when protein location is investigated individually, either fusion to a visible protein or immunological detection is used to confirm the expected locations.

Available location data for plant ABC proteins have been manually assembled, and a subcellular location or hypothetical location is reported in Table 1. Multiple databases were used in the assembly, starting with two aggregate predictors. The first is named the SUBcellular localization database for *Arabidopsis* proteins, version 3 (SUBA3) (Tanz et al. 2013). This program automatically uses large proteomic and GFP targeting information, as well as multiple targeting predictions. The second aggregate subcellular localization predictor is named MASCP Gator v1.1 (Joshi et al. 2011). Similar to SUBA3, Gator also compiles GFP targeting and proteomics data, but it does so by individually querying each individual database, giving more up-to-date results. To confirm the aggregate predictors, proteomic data were also collected from the Plant Proteome Database (PPDB), a frequently updated, manually curated proteomics database (Sun et al. 2009), and AtChloro, a chloroplast-specific manually curated proteomics database (Ferro et al. 2010).

Table 1 Subcellular locations of plant ABC proteins

		Locations								
Name	AtAGI	Curated		1	2	3	4	5	6	Synonyms	References
Plastid											
ABCG7	At2g01320	Ch	E		Ch (2)		PM	Ch	Ch	AtWBC7	Simm et al. (2013)
AtABCB26	At1g70610	Ch	IE		Ch (2)		Ch	Ch	Ch	AtTAP1	
AtABCB28	At4g25450	Ch	IE		Ch (5)		Ch	Ch	Ch	AtNAP8	Simm et al. (2013)
AtABCB29	At5g03910	Ch	IE		Ch (3)		Ch	Ch	Ch	AtATH12	Sudre (2009)
AtABCI10	At4g33460	Ch	IE		Ch (2)		Ch	Ch	Ch	NAP13	Garcia et al. (2004)
AtABCI11	At5g14100	Ch	IE		Ch (2)		Ch	Ch	Ch	NAP14	Simm et al. (2013), Shimoni-Shor et al. (2010)
AtABCI12	At3g21580	Ch	IE				Ch	Ch	Ch	CBIQ-like	
AtABCI13	At1g65410	Ch	IE		Ch (2)		Ch	Ch	Ch	TGD3	Lu et al. (2007), Roston et al. (2012)
AtABCI14	At1g19800	Ch	IE				Ch	Ch	Ch	TGD1	Xu et al. (2003, 2005a), Roston et al. (2012)
AtABCI15	At3g20320	Ch	IE		Ch (5)		Ch	Ch	Ch	TGD2	Lu and Benning (2009), Roston et al. (2011, 2012), Awai et al. (2006)
AtABCI6	At3g10670	Ch	Str		Ch (4)		Ch	Ch	Ch	SufC-like ,	Xu and Moller (2004)
AtABCI7	At1g32500	Ch	Str		Ch (2)		Ch	Ch	Ch	SufD-like,	Hjorth et al. (2005), Xu and Moller (2004)
AtABCI9	At5g44316	Ch	Str		Ch (2)		Ch	Ch (4)	Cy	SufB-like	Xu et al. (2005b)
AtABCD2	At1g54350	Ch			Ch (1)		Ch		Ch	AtPMP1	
AtABCF2	At5g09930	Ch					Ch			AtGCN2	Kato et al. (2009)
AtABCF5	At5g64840	Ch			Ch (1)		Ch		Ch	AtGCN5	Bayer et al. (2011), Kato et al. (2009)

(continued)

Table 1 (continued)

		Locations								
Name	AtAGI	Curated		1	2	3	4	5	6	Synonyms	References
ABCG23	At5g19410	Ch					Ch			AtWBC24	
AtABCI8	At4g04770	Ch			Ch (3)	C (1)	Ch	Ch	Ch	SufB-like, LAF6,	Xu et al. (2005b), Nagane et al. (2010)
AtABCC12	At1g30410	Ch?	Str		Ch (1)		PM	Ch (5)	V	AtMRP12/13	Wanke and Kolukisaoglu (2010)
AtABCB14	At1g28010	Ch?			Ch (1)		PM			AtPGP14, AtMDR12	
AtABCB15	At3g28345	Ch?			Ch (1)		PM	Ch (1)	PM	AtPGP15, AtMDR13	Kaneda et al. (2011a)
ABCG35	At1g15210	Ch?		PM	PM (4)		PM		Ch, PM	AtPDR7	Brautigam and Weber (2009)
Plasma Membrane											
AtABCA2	At3g47730	PM			PM (4)		PM		PM	AtATH1	
AtABCA3	At3g47740	PM			PM (1)		PM		PM	AtATH2	
AtABCA4	At3g47750	PM					PM			AtATH3	
AtABCA5	At3g47760	PM					PM			AtATH4	
AtABCA6	At3g47770	PM					PM			AtATH5	
AtABCA7	At3g47780	PM			PM (3)		PM		PM	AtATH6	
AtABCA8	At3g47790	PM					PM			AtATH7	
AtABCA9	At5g61730	PM			PM (3)		PM		PM	AtATH11	
AtABCA10	At5g61740	PM			PM (6)		PM		PM	AtATH14	
AtABCA11	At5g61690	PM					PM			AtATH15	
AtABCA12	At5g61700	PM					PM			AtATH16	
AtABCB1	At2g36910	PM		PM	PM (7)		PM		PM	AtMDR1	
AtABCB2	At4g25960	PM			PM (2)		PM		PM	AtMDR2	
AtABCB3	At4g01820	PM					PM			AtMDR3	
AtABCB4	At2g47000	PM		PM	PM (9)		PM		PM	AtMDR4	

AtABCB5	At4g01830	PM				PM		AtMDR5	
AtABCB6	At2g39480	PM	U	PM (6)		PM	PM	AtMDR6	
AtABCB7	At5g46540	PM				PM		AtMDR7	
AtABCB9	At4g18050	PM				PM		AtMDR9	
AtABCB11	At1g02520	PM	PM	PM (6)		PM	PM	AtMDR8	
AtABCB12	At1g02530	PM		PM (2)		PM	PM	AtMDR16	
AtABCB13	At1g27940	PM				PM		AtMDR15	
AtABCB16	At3g28360	PM				PM		AtMDR18	
AtABCB17	At3g28380	PM		PM (1)		PM	PM	AtMDR19	
AtABCB18	At3g28390	PM				PM		AtMDR20	
AtABCB19	At3g28860	PM	U	PM (4)	PM (1)	PM	PM, V	AtMDR11	
AtABCB20	At3g55320	PM		PM (4)		PM	PM, V	AtMDR14	
AtABCB21	At3g62150	PM		PM (8)		PM	PM	AtMDR17	
AtABCB22	At3g28415	PM		PM (2)		PM	PM	AtMDR21	
AtABCC4	At2g47800	PM	U	V (5)	PM (1)	PM	V	AtMRP4	Wanke and Kolukisaoglu (2010), Klein et al. (2004)
AtABCC7	At3g13100	PM		V (1)		PM	V	AtMRP7	Wanke and Kolukisaoglu (2010), Wojas et al. (2009)
AtABCC9	At3g60160	PM		PM (1)		PM	PM	AtMRP9	Wanke and Kolukisaoglu (2010)
AtABCC11	At1g30420	PM				PM		AtMRP11/12,	Wanke and Kolukisaoglu (2010)
AtABCC15	At3g60970	PM				PM		AtMRP15	Wanke and Kolukisaoglu (2010)
AtABCG1	At2g39350	PM		PM (3)		PM		AtWBC1	
AtABCG3	At2g28070	PM		PM (4)		PM	PM	AtWBC3	

(continued)

Table 1 (continued)

Name	AtAGI	Locations							Synonyms	References
		Curated	1	2	3	4	5	6		
AtABCG4	At4g25750	PM				PM			AtWBC4	
AtABCG5	At2g13610	PM				PM			AtWBC5	
AtABCG6	At5g13580	PM				PM			AtWBC6	
AtABCG8	At5g52860	PM				PM			AtWBC8	
AtABCG9	At4g27420	PM				PM			AtWBC9	
AtABCG10	At1g53270	PM				PM			AtWBC10	
AtABCG11	At1g17840	PM		PM (3)		PM		PM	COF1, DSO	
AtABCG12	At1g51500	PM		PM (2)		PM		PM	CER5	
AtABCG13	At1g51460	PM				PM		PM	AtWBC13	
AtABCG15	At3g21090	PM				PM			AtWBC15/22,	
AtABCG16	At3g55090	PM				PM			AtWBC16	
AtABCG17	At3g55100	PM				PM			AtWBC17	
AtABCG18	At3g55110	PM				PM			AtWBC18	
AtABCG20	At3g53510	PM				PM			AtWBC20	
AtABCG22	At5g06530	PM		PM (3)		PM		PM	AtWBC23	
AtABCG24	At1g53390	PM		PM (1)		PM		PM	AtWBC25	
AtABCG25	At1g71960	PM			PM (1)	PM			AtWBC26	
AtABCG26	At3g13220	PM			PM (1)	PM			AtWBC27	
AtABCG27	At3g52310	PM		PM (1)		PM		PM	AtWBC28	
AtABCG28	At5g60740	PM				PM			AtWBC29	
AtABCG29	At3g16340	PM		PM (2)		PM		PM	AtPDR1	
AtABCG30	At4g15230	PM				PM			AtPDR2	Badri et al. (2009)
AtABCG31	At2g29940	PM		PM (1)		PM		PM	AtPDR3	
AtABCG32	At2g26910	PM		V (1)		PM		V	AtPDR4	Badri et al. (2009)
AtABCG33	At2g37280	PM		PM (2)		PM		PM	AtPDR5	
AtABCG34	At2g36380	PM	PM	PM (5)		PM		PM	AtPDR6	

AtABCG36	At1g59870	PM	U	PM (11)	PM (1)	PM		PM	PEN3, AtPDR8	Simm et al. (2013), Xin et al. (2013), Kobae et al. (2006), Badri et al. (2012)
AtABCG37	At3g53480	PM	PM	PM (1)		PM		PM	AtPDR9/12	Badri et al. (2012)
AtABCG38	At3g30842	PM		PM (1)		PM			AtPDR10	
AtABCG39	At1g66950	PM		PM (1)		PM			AtPDR11/13	
AtABCG40	At1g15520	PM		PM (1)	PM (1)	PM			AtPDR12	
AtABCG41	At4g15215	PM				PM			AtPDR13	
AtABCG42	At4g15233	PM				PM			AtPDR14	
Frag. of C	At1g71330	PM		PM (1)		Cy,PM		PM	NAP5	Badri et al. (2012)
Frag. of G	At2g37010	PM				PM		PM	NAP12	
AtABCB14	At1g28010	PM?		Ch (1)		PM			AtMDR12	
AtABCB15	At3g28345	PM?		Ch (1)		PM	C (1)	PM	AtMDR13	Kaneda et al. (2011b)
AtABCC6	At3g13090	PM?		V (1)		PM		V	AtMRP6	Wanke and Kolukisaoglu (2010)
AtABCC12	At1g30410	PM?		Ch (1)		PM	Ch (5)	V	AtMRP12, AtMRP13	Wanke and Kolukisaoglu (2010)
AtABCC13	At2g07680	PM?		V (1)		PM		V	AtMRP13/11,	Wanke and Kolukisaoglu (2010)
AtABCF1	At5g60790	PM?		PM (3)		Cy		PM	AtGCN1	Badri et al. (2012), Wohlbach et al. (2008)
AtABCF3	At1g64550	PM?		PM (1)		Cy		PM	AtGCN3	Badri et al. (2012), Zeng et al. (2011), Kato et al. (2009)
AtABCG14	At1g31770	PM?				PM,Pe			AtWBC14	
AtABCG35	At1g15210	PM?	PM	PM (4)		PM		Ch, PM	AtPDR7	Brautigam and Weber (2009)
Vacuole										
AtABCA1	At2g41700	V		V (1)		PM		V	AtAOH	

(continued)

Table 1 (continued)

		Locations								
Name	AtAGI	Curated	1	2	3	4	5	6	Synonyms	References
AtABCB27	At5g39040	V	~V	V (4)	V (1)	V		V	ALS1	
AtABCC1	At1g30400	V	V	V (5)	V (1)	V		V	AtMRP1	Wanke and Kolukisaoglu (2010)
AtABCC2	At2g34660	V	V	V (3)		V		V	AtMRP2	Wanke and Kolukisaoglu (2010)
AtABCC3	At3g13080	V	~V	V (2)		V		V	AtMRP3	Wanke and Kolukisaoglu (2010)
AtABCC5	At1g04120	V	V	V (2)		V		V	AtMRP5	Wanke and Kolukisaoglu (2010), Badri et al. (2012)
AtABCC7	At3g13100	V		V (1)		PM		V	AtMRP7	Wanke and Kolukisaoglu (2010), Wojas et al. (2009)
AtABCC8	At3g21250	V	~V	V (2)		PM		V	AtMRP8	Wanke and Kolukisaoglu (2010), Jaquinod et al. (2007)
AtABCC10	At3g59140	V		V (1)		PM		V	AtMRP10/14	Wanke and Kolukisaoglu (2010), Jaquinod et al. (2007)
AtABCC14	At3g62700	V	U	V (5)		V		V	AtMRP14/10	Wanke and Kolukisaoglu (2010)
AtABCG19	At3g55130	V				PM		V	AtWBC19	Mentewab and Stewart (2005)
AtABCG43	At4g15236	V		V (1)		N,PM		V	AtPDR15	
AtABCC6	At3g13090	V?		V (1)		PM		V	AtMRP6	Wanke and Kolukisaoglu (2010)
AtABCC12	At1g30410	V?		Ch (1)		PM	Ch (5)	V	AtMRP12/13	Wanke and Kolukisaoglu (2010)

AtABCC13	At2g07680	V?		V (1)		PM		V	AtMRP13/11,	Wanke and Kolukisaoglu (2010)
Mitochondria										
AtABCB10	At1g10680	M		M (1)		PM		M	AtMDR10	
AtABCB23	At4g28630	M			M (1)	M			STA2, AtATM1	Chen et al. (2007)
AtABCB24	At4g28620	M		M (1)	M (1)	M		M	AtATM2	Chen et al. (2007)
AtABCB25	At5g58270	M	M, Ch	Ch (2)	M (2)	M	Ch, M	M	STA1, AtATM3	Chen et al. (2007); Bernard et al. (2009)
AtABCI1	At1g63270	M				M			ccmA-like, NAP10	Rayapuram et al. (2007)
AtABCI2	Atmg00110	M				M			ccmB-like	Rayapuram et al. (2007)
AtABCI3	Atmg00900	M				M			ccmC-like	
AtABCI4	At2g07681	M				M			ccmC-like	
AtABCI5	At2g07771	M				M			ccmC-like	
Peroxisome										
AtABCD1	At4g39850	Pe		PM (2)		Pe		Pe	PXA1, CTS, PED3, ACN2, AtPMP2	
AtABCG14	At1g31770	Pe?				PM,Pe			AtWBC14	
Cytosol										
AtABCE1	At3g13640	Cy				Cy			AtRLI1	
AtABCE2	At4g19210	Cy		PM (2)		Cy		PM	AtRLI2	Kougioumoutzi et al. (2013)
AtABCE3	At4g30300	Cy				Cy			AtNAP15	
AtABCF4	At3g54540	Cy				Cy			AtGCN4	
AtABCG21	At3g25620	Cy				Cy			AtWBC21	
AtABCI16	At2g37330	Cy							AtSTAR2, ALS3	Huang et al. (2009, 2010)

(continued)

Table 1 (continued)

		Locations								
Name	AtAGI	Curated	1	2	3	4	5	6	Synonyms	References
AtABCI17	At1g67940	Cy		V (1)		Cy		PM, V	AtSTAR1	Huang et al. (2009, 2010)
AtABCI19	At1g03905	Cy			Cy (1)	Cy			NAP4	Marin et al. (2006)
AtABCI20	At5g02270	Cy			Cy (1)	Cy			NAP9	Marin et al. (2006)
AtABCI21	At5g44110	Cy				Cy			POP1, NAP2	Marin et al. (2006)
AtABCF1	At5g60790	Cy?		PM (3)		Cy		PM	AtGCN1	Badri et al. (2012), Wohlbach et al. (2008)
AtABCF3	At1g64550	Cy?		PM (1)		Cy		PM	AtGCN3	Badri et al. (2012), Kato et al. (2009), Zeng et al. (2011)
Miscellaneous										
AtABCI18	At1g03900	N				N				
AtABCG2	At2g37360	n.d.							AtWBC2	

Categories from left, systematic name of the protein (Name), Arabidopsis accession number (AtAGI), curated location (Curated), location predictors: *phenoDisco* (1), Gator proteomics (2), Gator GFP (3), SUBA3 (4), AtChloro (5), PPDB (6). Unpredictable proteins from *phenoDisco* are labeled U. Those with questionable predictions have a ~ preceding the location. Numbers in parentheses in Gator analyses represent the number of studies reporting that location. Numbers in parentheses in AtChloro refer to the number of spectral counts and have been included only when spectral counts were low. Common names for proteins are given under (Synonyms), though not all have been included for space reasons. References (Ref) used in manual curation are given. Curated proteins assigned to more than one subcellular location are indicated with a ? after the location. Subcellular locations are abbreviated as follows: *Ch* plastid, *PM* plasma membrane, *V* vacuole, *M* mitochondria, *Pe* peroxisome, *Cy* cytosol, *N* nucleus, *n.d.* no data, *E* plastid envelope membranes, *IE* plastid inner envelope membrane, *Str* stroma

Additionally, data from a new method of predicting localization based on analysis of organelle-specific protein clusters among whole cell proteomics experiments with fractionated organelles, *phenoDisco*, was included (Breckels et al. 2013). These data were organized for each gene, and then conflicts between programs were resolved by manual search of available publications. If no publications were available, conflicts were resolved in favor of physical data (i.e., proteomics rather than predictions). Finally, if no "best" subcellular location could be identified, the proteins were assigned more than one location with a question mark denoting the possible unsuitability of the prediction (Table 1).

For plastidic ABC proteins, suborganellar locations are also given where possible. These are derived from either individual investigation or proteomic evidence.

3 Plastidic Fe–S Cluster Assembly by the SUFs

Multiple plastidic proteins require iron (Fe)–sulfur (S) clusters, particularly those involved in the light reactions of photosynthesis but also those in other areas of metabolism. Some of the more abundant examples of plastidic Fe–S cluster proteins include the cytochrome b_6f complex, photosystem I, glutamate synthase, and 5′-adenylylsulfate (APS) reductase (Balk and Pilon 2011). Thus, Fe–S cluster assembly and transport are essential processes in many types of plastids. A bacterial and cyanobacterial system responsible for Fe–S cluster formation has been named "SUF" for "sulfur mobilization" and seems to have been maintained in plastids (Xu and Moller 2004). The SUF system requires multiple proteins to generate Fe–S clusters in a controlled manner. In bacteria, gene clusters encode 6 or more genes necessary for sulfur assimilation, and in land plants, additional accessory proteins appear to be involved. For further information and a complete discussion of Fe–S cluster metabolism, the following reviews are suggested (Couturier et al. 2013; Balk and Pilon 2011). Here we will focus only on the roles of the three ABC proteins involved: AtABCI8/SUFB/NAP1/LAF6/AtABC1 (At4g04770), AtABCI6/SUFC/NAP7 (At3g10670) and AtABCI7/SUFD/NAP6 (At1g32500), see Fig. 1. SUFB does have a second possible homolog in Arabidopsis, AtABCI9 (At5g44316). Initial studies of SUFB in Arabidopsis attempted to investigate this gene as well, but failed to identify transcripts, therefore it has not been studied further (Xu et al. 2005b).

Unlike most ABC transporter components, some NBD-containing ABC family proteins are not associated with TMDs or SBDs and do not perform transport functions (Higgins 1992). In plants, these have been called non-intrinsic ABC proteins (NAPs) (Sanchez-Fernandez et al. 2001), and SUFB, C, and D are among them. *SUFC* and *SUFD* are necessary for normal plant development as T-DNA insertions disrupting gene functions cause embryo-lethality in Arabidopsis (Hjorth et al. 2005; Xu and Moller 2004). Nonlethal alleles of *SUFB* have been isolated, and these plants accumulate chlorophyll precursors or have impaired chlorophyll degradation (Nagane et al. 2010; Moller et al. 2001). Because Fe–S

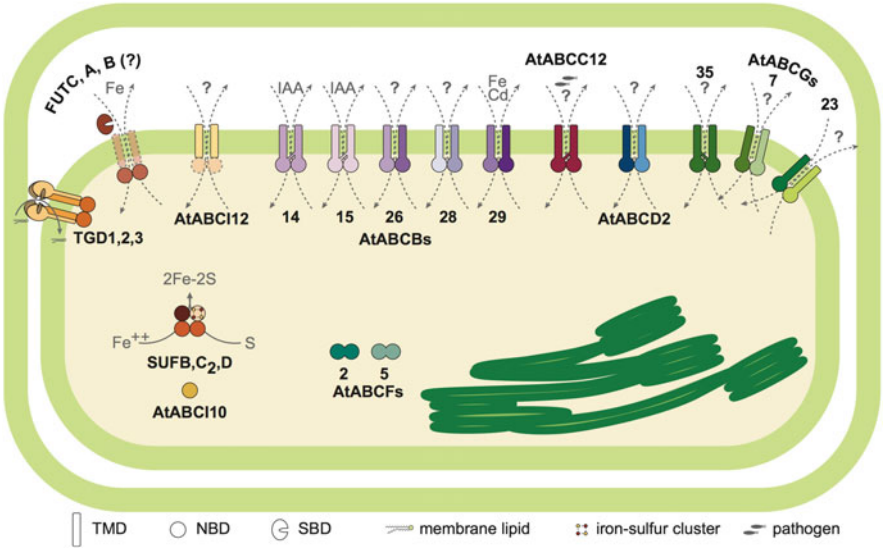

Fig. 1 ABC proteins identified as plastidic in Table 1 are shown. The structures of the transporters or putative transporters are indicated; fused domains are shown in the same *shade*. (i.e., full transporters appear one shade, half transporters in two, etc.) Families are grouped by *color*. *Solid lines* indicate known proteins or substrates; *dashed lines* indicate putative subunits or substrates. Note that the orientation of most transporters relative to the membrane is unknown, excepting TGD1,2,3, therefore putative substrates are shown with entry and exit lines

cluster containing proteins are required for chlorophyll metabolism, the mutant phenotypes along with similarities of plant *SUF*s to bacterial Fe–S cluster forming *SUF*s, strongly imply that plant *SUF*s have a role in Fe–S cluster formation in plastids.

In bacteria, SufB, C, and D form two distinct complexes, the $SufBC_2D$ complex (Wollers et al. 2010) and the $SufB_2C_2$ complex (Saini et al. 2010). Whether separate in vivo roles exist for the two complexes is still being investigated (Chahal and Outten 2012), though it is known that all three proteins (SufB, SufC, and SufD) are necessary for Fe–S cluster assembly. Mobilized sulfur is passed to SufB by SufE. The transfer is only completed if SufC is also present (Layer et al. 2007). Iron is then transferred to SufB in a mechanism requiring both SufC and SufD. SufC is assumed to provide energy for this process by its ATPase activity, while the role of SufD is unclear, but required (Saini et al. 2010). The Fe–S cluster is then assembled on SufB, possibly using the reducing power of flavin adenine, which is also bound by SufB (Wollers et al. 2010). The assembled Fe–S cluster is finally transferred to acceptor proteins, either directly by action of the SufBCD complex or through the action of accessory proteins, in a manner which has yet to be understood.

Current evidence suggests that the plant SUFs catalyze Fe-S cluster formation similarly to their bacterial counterparts. SUFC from Arabidopsis was shown to have ATPase activity in vitro and to partially complement *sufc*-deficient *E. coli* (Xu and

Moller 2004). Arabidopsis SUFB contains a completely conserved amino acid sequence with *E. coli* SufB in the proposed binding sites for flavin adenine and Fe–S cluster assembly (Balk and Pilon 2011). Furthermore, Arabidopsis has one or more plastidic homologs of all the genes required for the Suf system of Fe–S cluster formation and mobilization (Couturier et al. 2013). In addition to these individual functional similarities to their bacterial counterparts, Arabidopsis SUFB, SUFC, and SUFD have been shown to interact, though the stoichiometry of each subunit in a functional complex has not been investigated yet (Xu et al. 2005b; Xu and Moller 2004). There are also hints that the plant SUFBCD complex deviates from its bacterial homologs. In *E. coli*, SufB does not have detectable ATPase activity, while in Arabidopsis, SUFB has iron-stimulated ATPase activity (Xu et al. 2005b). Similarly, in *E. coli*, the SUFs are not required for growth except under conditions of oxidative stress (Nachin et al. 2003), whereas in Arabidopsis they are always required. It should be noted that plant SUFs can also respond to stress conditions, as it was recently shown in rice that plastidic Fe–S cluster formation responds with marked tissue specificity to heavy metal stress (Liang et al. 2013). However, the rationale for increased dependence on the SUF system in plants remains unclear. What is clear is that the plant ABC proteins are playing a similar yet subtly different role than their bacterial homologs. Further investigation, particularly into the role of the ATPase activity of SUFB, will help to distinguish the plant SUFs from bacterial counterparts.

4 Iron Transport into Plastids

Import of iron is necessary to supply Fe^{2+} and Fe–S cluster cofactors within the plastid. Direct import of Fe^{2+} by the plastid inner envelope membrane has been demonstrated using chloroplasts isolated from pea plants (Shingles et al. 2002). However, because free iron ions tend to promote formation of highly reactive oxygen species, transport and storage of iron must be tightly regulated. In cyanobacteria, multiple families of transporters have been implicated in iron transport, including the ABC transporter FutABC.

In *Synechocystis sp PCC6803*, the multipartite FutABC transporter is encoded by four genes. *futB/slr0327* encodes the TMD, *futC/sll1878* the NBD, and there are two highly similar genes encoding SBDs, *futA1/slr1295* and *futA2/slr0513*. The function of *futABC* genes in iron transport was inferred because transcripts of these genes were highly upregulated during iron stress, and because *Synechocystis* strains lacking *fut* gene expression grew poorly on iron-depleted medium (Katoh et al. 2001).

AtABCI11/NAP14 (At5g14100) of Arabidopsis was found to be similar to FutC (31.5 % identity at the protein level) (Larkin et al. 2007a). Arabidopsis T-DNA insertion alleles that disrupt *AtABCI11* expression could not grow on soil, only on sucrose-supplemented complete medium. Even on this medium, they were small and white in appearance. Biomass of the mutant was reported to increase when

grown on low-iron medium, consistent with a toxic level of Fe^{2+} accumulating in the cytosol in iron-rich medium and supporting its possible role in Fe^{2+}transport and sequestration in the plastid (Shimoni-Shor et al. 2010). However, multiple data are also in opposition to it being part of a functional plastidic Fe^{2+} transporter:

First, transcripts of *AtABCI11* do not respond to iron stress (Shimoni-Shor et al. 2010; Hruz et al. 2008), which is expected of a required iron transporter. The multiple families of metal ion transporters have been shown to transport different metal ions in different cyanobacterial species. For example, in *Anabaena*, the Fut transporter responds more strongly to copper ions than to iron (Stevanovic et al. 2012). So it is possible that AtABCI11 transports another metal and accumulation of multiple metal ions, including copper, was seen in *abci11* plants (Shimoni-Shor et al. 2010). Plastidic copper transporters are already known, i.e., PAA1, PAA2 (Puig and Penarrubia 2009), HMA1 (Seigneurin-Berny et al. 2006), but this does not exclude AtABCI11 as a possible copper transporter. Alternatively, the accumulation of metal ions in *abci11* plants could simply be a secondary phenotype related to decreased growth.

Second, *AtABCI11* is one gene of a three-gene transporter. Plastidic homologs of FutB are not identifiable in plant species, either with multiple rounds of NCBI DELTA- and PSI-BLASTs or by co-regulation at the transcriptional level (Hruz et al. 2008). A possible homolog of FutA was revealed through similarity searches to be At1g31410, which encodes a plastid protein with a substrate binding domain recently shown to have a knockout phenotype similar to that of *abci11* (Tameshige et al. 2013). Given the similarity of the At1g31410 encoded protein to ABC substrate binding domains, the name AtABCI22 is suggested. Interestingly, *AtABCI22* may be coexpressed with a high Pearson's Correlation Coefficient (PCC 0.75) with *AtABCD2* (Hruz et al. 2008). Pearson's Correlation Coefficients are measurements of the expression similarity between two genes, they do not necessarily indicate a functional relationship, but are likely to indicate shared responses to stimuli. Values range from 0 to 1 with higher values indicative of more significant correlation (Obayashi and Kinoshita 2009). Thus, similar expression profiles of *AtABCD2* and *AtABCI22* do not indicate the likelihood of direct interaction between AtABCD2 and AtABCI22, especially as AtABCD2 has its own NBD and TMD domains (see Sect. 9). Rather, transcripts of AtABCI22 seem to be expressed in response to various stresses, and during different stages of development similarly to transcripts of AtABCD2, a correlation which corroborates its location as a plastid protein. However, even if *AtABCI22* is considered a potential FutA homolog, a homolog for FutB may not exist, see Fig. 1. Needless to say, without FutA, FutB, and FutC homologs, AtABCI11, and AtABCI22 are unlikely to form a functional iron transporter.

Finally, and possibly most importantly, the study of Fe^{2+} transport using isolated pea chloroplasts showed that transport was stimulated not by ATP hydrolysis, but by a proton gradient (Shingles et al. 2002). This indicates that even if a FutB homolog were found in Arabidopsis, it is unlikely that the plastidic FUT system would be the major iron importer.

Because of the need for both iron transport and regulation in the plastid, it is possible that the FUT system could have evolved from a transport mechanism into a control mechanism. Future work on the FUT system in plants should determine whether an entire FUT transporter is available and then more finely characterize its function.

5 Lipid Transport Through the Chloroplast Envelopes by TGDs

Plastids have a unique complement of membrane lipids including glycolipids monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and sulfoquinovosyldiacylglycerol (SQDG) (Block et al. 2013). These lipids are conserved throughout photosynthetic organisms including algae and cyanobacteria. Glycolipids are believed to have evolved in response to limiting phosphate and/or a role in photosynthesis as they are found in crystal structures of photosytems (Guskov et al. 2009). While cyanobacteria synthesize all of their lipids in the cell, and algae may to be able to synthesize plastidic lipids entirely in the plastid (Giroud et al. 1988), land plants have evolved a more complex system requiring lipid import from the ER (Heinz and Roughan 1983). In this system, fatty acyl groups are synthesized in the plastid stroma, exported to the ER where they are incorporated into phospholipids, and then transported back to the plastid where they are metabolized to plastidic lipids in the envelope membranes. This process has been recently reviewed (Andersson 2013; Wang and Benning 2012); here we will focus on lipid transport from the plastid outer envelope membrane to, and perhaps through, the inner envelope membrane which involves an ABC transporter. This transporter is composed of three proteins: AtABCI14/TGD1 (At1g19800), AtABCI15/TGD2 (At3g20320), and AtABCI13/TGD3/NAP11 (At1g65410).

TGD1, TGD2, and TGD3 are most similar in structure to a multipartite ABC transporter as separate genes encode the TMD (TGD1), NBD (TGD3), and the substrate-binding domain (SBD, TGD2). Interestingly, the TGD2 substrate-binding domain is anchored with a transmembrane domain, an unusual feature only commonly found in Archaea (van der Heide and Poolman 2002), though cyanobacterial homologs of TGD2 also have a transmembrane domain. It would be convenient to draw analogies between the TGD system and a well-established bacterial system; however, cyanobacterial homologs have not been well studied, and functional homologs in *Escherichia coli* have only recently been identified. Based on sequence similarity to both the plant TGD system and cholesterol transporters of Actinobacteria, an ABC transporter composed of MlaD (SBD), MlaE (TMD), and MlaF (NBD) was identified as necessary for maintenance of bacterial outer membrane lipid asymmetry (Malinverni and Silhavy 2009). Mutants without expression of one or more of the *mla* genes showed accumulation of phospholipids in the outer leaflet of the bacterial outer membrane. The authors hypothesize that the Mla

transporter serves to recycle phospholipids from the outer membrane by importing them to the inner membrane. However, direct evidence to support their hypothesis is not yet available.

In Arabidopsis, early work identified point mutants in *tgd1* and *tgd2* genes as part of a large screen to find repressors of a mutant in DGDG synthesis, *dgd1* (Xu et al. 2003; Awai et al. 2006). The *tgd* mutants not only produced additional DGDG; they also made a non-canonical lipid from which their name is derived: trigalactosyldiacylglyerol. The most relevant phenotypes of the *tgd1* mutant were reduced levels of ER-derived fatty acids in plastid lipids MGDG and DGDG, implying its role in ER to plastid lipid transport, and its high embryo lethality rate of approximately 50 %, which highlighted the importance of ER to plastid lipid transport (Xu et al. 2005a). Other phenotypes of *tgd1* were investigated in detail and included reduced chlorophyll levels, increased extraplastidic triacylglycerol accumulating in lipid droplets, increased levels of phosphatidic acid (PtdOH), and reduced ability of isolated chloroplasts to incorporate lipids supplied in vitro (Xu et al. 2003, 2005a). The phenotypes of *tgd2* were similar to those of *tgd1* (Awai et al. 2006). TGD3 was identified by screening T-DNA insertions in predicted plastidic NAPs for production of trigalactosyldiacylglycerol and lipid profiles similar to those of *tgd1* and *tgd2* mutants (Lu et al. 2007).

In addition to their mutant phenotypes, the TGD proteins have been investigated both individually and in their native complex in some detail. Current evidence shows that both TGD1 and TGD2 are integral membrane proteins of the plastid inner envelope membrane with the active SBD side of TGD2 facing the outer envelope membrane (Xu et al. 2003; Awai et al. 2006). TGD2 specifically binds PtdOH, a potential lipid substrate (Lu and Benning 2009). TGD3 is peripheral to the same membrane, on the stromal side, and a functional ATPase in vitro (Lu et al. 2007). Thus, the three TGDs are in the correct location and orientation and have the measured or predicted functionality to form an ABC transporter complex in the inner envelope which accepts substrates from the outer envelope membrane, see Fig. 1. They have been shown to interact and to form a single large complex in Arabidopsis (Roston et al. 2012). The subunit composition of the complex was unusual in that there are at least 8 copies of TGD2 per functional transporter. This varies from traditionally studied multipartite ABC transporters, which generally have only one or two SBD proteins bound per transporter (van der Heide and Poolman 2002). It is possible that the increase in SBDs is required to overcome the energetic barrier of moving hydrophobic lipids across the hydrophilic intermembrane space or to use cooperativity to increase binding to PtdOH which is a low-abundance substrate. It is also worth noting that the soluble region of TGD2 (with the transmembrane region removed) was shown to be capable of disrupting membranes in vitro (Roston et al. 2011). This is likely a necessary function for proteins that bind polar lipids in membranes, but it may provide a third rationale for additional TGD2 proteins per complex as more TGD2s imply more efficiency in disrupting membranes allowing the substrate lipid to be more easily extracted from its membrane.

There are at least two major questions which remain to be resolved regarding the function of TGD1, 2, and 3. Although the involvement of each TGD in ER to plastid lipid transport is apparent from the phenotypes of *tgd* mutants of Arabidopsis, it remains unclear from which envelope membrane leaflet the substrate is derived, and what type of substrate is actually transported. It has been hypothesized that TGD2 directly interacts with the plastid outer envelope membrane to extract a substrate (Roston et al. 2012). Alternatively, it is possible that TGD2 interacts with the outer leaflet of the inner envelope membrane, meaning the transporter would transport the substrate only across the inner envelope membrane, which is also known as "flippase" activity. Other subfamilies of ABC transporters that transport lipids in mammalian cells have flippase activity (Coleman et al. 2013), although these transporters do not associate with a SBD-containing protein comparable to TGD2. Regarding the second question of the substrate transported, PtdOH has been proposed to be the substrate based on binding of TGD2 and increased PtdOH levels in the *tgd* mutants. However, in vitro TGD2 only binds to membranes containing more than 20 mol percent of PtdOH (Lu and Benning 2009). These concentrations do not exist in vivo (Dubots et al. 2012). Alternate hypotheses have suggested that TGD2 binds to PtdOH for activation or to promote interaction with a membrane surface while other substrates are transported, possibly in a less discriminatory way. Future work, especially reconstituting a functional TGD1, 2, 3 complex in vitro, may provide answers to these questions.

6 The ABCB Subfamily in Plastids

The ABCB subfamily of proteins contains two types of fused ABC transporters: both "half" and "full" transporters, meaning that a single ABCB gene can encode a TMD and NBD which can homo- or heterodimerize to make a full transporter or that it encodes fusions of two TMDs and two NBDs which can make a full transporter in a single polypeptide. Representatives of several major ABCB subgroups have been identified in the plastid by proteomics: The P-glycoprotein (PGP) / multidrug resistance (MDR) subgroup of full transporters is represented by AtABCB14/AtPGP14/AtMDR12 (At1g28010) and AtABCB15/AtPGP15/AtMDR13 (At3g28345). The transporter associated with antigen processing (TAP) subgroup of half transporters is represented by AtABCB26/AtTAP1 (At1g70610), AtABCB28/AtNAP8 (At4g25450), and AtABCB29/AtATH12 (At5g03910), see Fig. 1. Of the TAP representatives, AtABCB29 was identified as being most similar to the prokaryotic lipid A-like exporter (LLP) subgroup (Verrier et al. 2008).

Of the five plastidic ABCB transporters, those in the PGP/MDR subgroup have been best studied, as they were identified in a search for transporters responsible for export of lignin precursors, i.e., phenylpropanoids (Kaneda et al. 2011b). Among the transporters identified by coexpression with phenylpropanoid biosynthetic genes were several ABC transporters, including *AtABCB14* and *AtABCB15*.

Promoter activity of each gene was demonstrated through GUS promoter fusions to be high in the vasculature and in guard cells, a likely expression pattern which is corroborated by publicly available microarray data (Hruz et al. 2008; Kaneda et al. 2011b). Arabidopsis with T-DNA insertions that disrupt expression of *AtABCB14* or *AtABCB15* did not have lignin defects but did have altered indoleacetic acid (IAA) transport properties. The study stopped short of showing a direct role in IAA transport for AtABCB14 or AtABCB15, though this has been done for highly similar ABCB subfamily members (Yang and Murphy 2009). Circumstantial evidence suggests that IAA accumulates in a subcompartment of the cell under certain conditions (Davies 2010), and the plastid has been observed multiple times in multiple plant species to contain large amounts of IAA (Sandberg et al. 1990; Aloni et al. 2003). Thus, it seems possible that AtABCB14 and AtABCB15 may have roles in either sequestering IAA inside the plastid or in releasing it from such sequestration. It should be noted that many mammalian transporters in the ABCB subfamily, and particularly in the PGP/MDR subgroup, were discussed to own broad substrate specificity. It follows that a direct role in IAA transport would not preclude transport of other small molecules, and this may not be the only role of AtABCB14 and AtABCB15.

Reports delineating the roles of the AtABCB26 and AtABC28 transporters have not been published at the time of writing, and available bioinformatic data alone can only shed limited light on their functions. *AtABCB26* is expressed much higher in the sperm cell than in any other tissues, although it has been detected at medium to low levels in multiple tissues (Hruz et al. 2008). If true, this observation is of interest as sperm cells, the two cells which travel with the vegetative pollen tube as nuclei before reforming into separate cells, have not been observed to have plastids (Yu et al. 1989), indicative of a possible non-plastidic function of AtABCB26. At the transcriptional level, *AtABCB26* does seem to be co-regulated with genes encoding other plastid-targeted proteins, including *AtABCF5* (PCC 0.73), which provides correlative evidence for plastid localization (Hruz et al. 2008). It is possible that AtABCB26 functions in more than one subcellular compartment. AtABCB26 appears to form a small clade with homologs from both corn (ZmABCB27) and rice (OsABCB24) (Pang et al. 2013), likely indicating functional conservation in these species. Transcription of the rice homolog is increased in response to drought and salt stresses (Xing et al. 2012). Unfortunately, gene expression in sperm cells has not been reported in rice. Information about the substrate(s) of AtABCB26 or its homologs is lacking. Arabidopsis containing T-DNA insertions disrupting expression of *AtABCB26* were investigated as a part of the Chloroplast 2010 project, but no robust phenotypes were identified (Ajjawi et al. 2010). Within Arabidopsis, AtABCB26 has a fairly close homolog; AtABCB27/TAP2 (At5g39040) with 34.5 % identity in the functional region of the proteins (Larkin et al. 2007a). AtABCB27 is a vacuolar protein involved in aluminum toxicity resistance (Larsen et al. 2007), thus we could hypothesize that AtABCB26 is involved in metal transport in the plastid. However, given the broad substrate specificity of the ABC transporters, little weight could be placed on such a hypothesis. The strongest conclusions that can be drawn are that AtABCB26 is

likely in the plastid during most of the plant life cycle (proteomic data and found to be co-regulated with multiple plastid genes), where it performs as yet undiscovered, but likely conserved, functions.

A very similar conclusion can be reached for AtABCB28. Like the Arabidopsis mutant *abcb26*, Arabidopsis lacking functional *AtABCB28* have no phenotype uncovered by the Chloroplast 2010 project (Ajjawi et al. 2010). *AtABC28* seems to have moderately high expression in most tissues with the highest expression in seeds. It is most closely co-regulated with At5g51020 (PCC 0.69), which encodes CRL a plastid envelope AAA-ATPase involved in regulating leaf morphogenesis (Hruz et al. 2008). It has no near homologs in Arabidopsis, but it forms a small clade with those from rice (OsABCB26) and corn (ZmABCB23) (Pang et al. 2013), indicative of functional conservation in land plants. In rice, the homologous gene has high expression in ovaries and shoot tissues, which decreases during drought and salt stresses. AtABCB28 is likely to be a plastidic transporter providing an important, but not essential, function under the conditions tested.

AtABCB29 is the closest homolog in Arabidopsis to the lipid A transporter from prokaryotes. Lipid A is the lipid portion of lipopolysaccharides, the major component of the outer membrane of gram-negative bacteria. Although lipid A has not been detected in plants, Arabidopsis has been shown to encode genes homologous to those of the entire lipid A biosynthetic pathway (Li et al. 2011). At least one of the Arabidopsis lipid A-synthesizing proteins, AtLPXA, can complement *E. coli* with a temperature sensitive version of its homolog, *lpxa* (Joo et al. 2012). Because lipid A has not been discovered in plants, it is hypothesized that the homologous lipid A synthesis pathway has alternate substrates in plants, but these remain undiscovered at this time. It is possible that AtABCB29 is part of a transport mechanism for these undiscovered lipid A-like products or that it transports another plastid lipid, many of which are trafficked by currently unknown mechanisms (Andersson 2013). *AtABCB29* is expressed in multiple tissues, most highly in leaves and cotyledons and lowest in sperm cells (Hruz et al. 2008). It is coexpressed with At5g38660 at a high PCC (0.84). At5g38660 is a gene involved in acclimation of photosynthesis to the environment (*APE1*), and its coexpression with *AtABCB29* is indicative that these genes have similar responses to photosynthetic stresses. AtABCB29 forms a small clade with presumed homologs from rice (OsABCB27) and corn (ZmABCB30). Interestingly, AtABCB29 was investigated for possible metal transport into chloroplasts (Sudre 2009). In the study, AtABCB29 is confirmed to be a plastidic protein by fusion with YFP. Homozygous knockouts in Arabidopsis are shown to have normal physiology unless they are grown on cadmium or on low iron, and the AtABCB29 protein is shown to reduce cadmium toxicity in yeast. These data support a direct role of AtABCB29 in metal transport in chloroplasts, although the direction of transport and the specificity of AtABCB29 for cadmium versus other metals are not yet known. Again, this role may not preclude additional roles of AtABCB29.

7 AtABCC12

ABC proteins in the C subfamily are full size transporters with two TMDs and two NBDs encoded by a single gene, though many proteins in the family have an additional, third TMD at the N-terminus. A subset of the ABCC family members is notable because they do not act as transporters in spite of having all the domains necessary to do so (Verrier et al. 2008). In Arabidopsis, there is only one ABCC family member for which multiple plastidic peptides have been identified, AtABCC12/AtMRP12/AtMRP13 (At1g30410), see Fig. 1. AtABCC12 peptides have also been identified in the vacuole, which is the compartment where most of the ABCC family members are located. Therefore, the location of AtABCC12 remains to be firmly established; it may be dual-localized to both compartments, or one of the two current locations may have been identified erroneously.

AtABCC12 has not been studied, and the significance of its possible plastid localization is unclear. It has a very close homolog in Arabidopsis, AtABCC11 (90.3 % identity at the amino acid level), though investigation of *AtABCC11* expression revealed that it does not have full-length transcripts and may be a pseudogene (Kamimoto et al. 2009). In comparison, *AtABCC12* is expressed at moderate levels in most plant tissues with slightly higher expression in the roots than shoots (Hruz et al. 2008; Kamimoto et al. 2009). It does seem to be upregulated in response to pathogens, particularly *Pseudomonas syringae pv. tomato* (Wanke and Kolukisaoglu 2010). Possibly because of the pervasiveness of *AtABCC12* gene expression, no closely co-regulated genes have been identified (highest PCC 0.57). Most of the studied ABCC family members in plants are involved in sequestration of glutathione-S conjugate and other metabolites into the vacuole, ultimately providing strong pathogen resistance. They are also involved in chlorophyll catabolism (Lu et al. 1998), and it is possible that this effort would be assisted by a plastid-localized chlorophyll-metabolite exporter. If such a function gave an adaptive advantage, AtABCC12 would be expected to form a conserved clade among other land plant species; instead AtABCC12 clusters more closely to other Arabidopsis ABCCs (Pang et al. 2013). Currently, the relevance of AtABCC12 to plastid biology, if any, remains ambiguous.

8 AtABCD2

The ABCD subfamily is one of the smallest ABC subfamilies in Arabidopsis with only two members. The first of these is AtABCD1/AtPXA1/AtPMP2 (At4g39850), a well-studied peroxisomal protein responsible for the import of fatty acids and fatty acid-derived signaling molecules to the peroxisome (Baker et al. 2006). The second member of the D subfamily is AtABCD2/AtPMP1 (At1g54350), a fused ABC half transporter which is located in the plastid, see Fig. 1 and chapter "Function of ABCBs in Light Signaling."

Based on the roles of AtABCD1, biological functions have been hypothesized for AtABCD2, particularly fatty acid export from the plastid (Jouhet et al. 2007) but also export of hormone precursors like oxo-phytodienoic acid (OPDA) and redistribution of fat-soluble secondary metabolites like carotenoids and tocopherols. Each of these functions is believed to be essential for plant growth. Correspondingly, a relatively large number of investigators have ordered one or more of the available Arabidopsis mutant lines homozygous for disruption of *AtABCD2* from The Arabidopsis Information Resource. However, no publications have yet referenced these lines, and in the Chloroplast 2010 based characterization of two of these lines, no strong phenotypes were observed under the conditions tested (Ajjawi et al. 2010). From other databases, it is known that *AtABCD2* transcription levels are relatively higher in shoots, lower in roots, and it may be slightly responsive to dark treatment (Hruz et al. 2008). Several genes have a high likelihood of co-regulation at the transcriptional level, including At2g30390 (PCC 0.85) encoding ferrochelatase 2, and At5g42310 (PCC 0.85) encoding a plastid pentatricopeptide repeat containing protein. This transcriptional information is not particularly revealing other than that the correlation to other plastid genes provides corroboration for plastid localization of AtABCD2. Perhaps the best conclusion which can be drawn from available data is that none of the current predictions of AtABCD2 function are correct, and alternative hypotheses for the function of this protein are awaiting verification.

9 AtABCF2 and 5

The F subfamily of ABC proteins is different from both multipartite prokaryotic ABC proteins that have only one domain per gene and typical fused ABC transporters that have TMDs and NBDs in a single gene. Instead, ABCF subfamily genes encode two fused NBDs. They are conserved throughout eukaryotes, and though a role in transport has yet to be discovered, at least one ABCF protein has been shown to have an important regulatory role. In yeast and humans, ABCF1 mediates cellular translation activity through activation of the eIF-2 alpha kinase (Tyzack et al. 2000; Marton et al. 1997).

In Arabidopsis, two highly similar ABCF proteins are expected to be located in the plastid: AtABCF2/AtGCN2 (At5g09930) and AtABCF5/AtGCN (At5g64840) see Table 1 and Fig. 1. AtABCF2 and AtABCF5 are highly similar at both the DNA (81.7 % identity) and protein (82.2 % identity) level (Larkin et al. 2007b) and the genes reside in a duplicated region of chromosome 5 in Arabidopsis (Arabidopsis Genome 2000). However, the two genes seem to have discernible expression profiles. A recent investigation into promoter activity of genes from the AtABCF subfamily using GUS fusion constructs showed that *AtABCF5* was expressed in most plant tissues, while *AtABCF2* was expressed primarily in the anthers (Kato et al. 2009). Available microarray data confirm these differences and add the observation of *AtABCF2* expression in guard cells (Hruz et al. 2008). Arabidopsis

T-DNA insertion mutants disrupting expression of either gene did not show phenotypes during normal growth conditions. It is possible that *AtABCF2* and *AtABCF5* expression patterns, although appearing unique in wild-type plants, were altered enough in the mutant to allow functional complementation by the remaining gene, which would imply identical function of the proteins. Corroboration for identical functions comes from phylogenetic studies with corn and rice in which AtABCF2 and AtABCF5 are shown to be more highly related than their homologs, ZmABCF7 and OsABCF6 (Pang et al. 2013). As an alternate explanation for the lack of visible phenotypes in the Arabidopsis mutants, AtABCF gene functions may only be required during stress conditions (Kato et al. 2009). This would be similar to the situation in yeast, where ABCF1 only modulates translation under amino acid starvation conditions (Hinnebusch 1997).

Very little additional information can be mined from bioinformatic analyses. *AtABCF5* seems to be coexpressed with a high Pearson's correlation coefficient (PCC, 0.88) to At5g67030 (Hruz et al. 2008). This gene product, called ZEP or ABA1, is an ABA-responsive zeaxanthin epoxidase associated with the chloroplast thylakoid membrane. *AtABCF2* has a high PCC (0.87) with At5g17830 when tissue specific microarray data are analyzed (Hruz et al. 2008), however this gene has not been studied. If we assume that, like their non-plant family members, plant ABCFs are involved in regulation in response to stress, then there are certainly many highly regulated processes in the plastid with unknown regulatory subunits. However, there are too few data to predict which process is affected. Considering the increasing focus on stress biology, potential regulators of stress responses, including the ABCFs, are likely to become increasingly relevant.

10 The ABCG Subfamily in Plastids

The ABCG subfamily consists of "reverse orientation" transporters in which a NBD precedes the TMD in the gene sequence of a fused transporter. There are two major ABCG subgroups, the white–brown complex (WBC) half-transporter subgroup and the pleiotropic drug resistance (PDR) full-transporter subgroup. The WBC subgroup is found in all types of organisms, but the PDR subgroup is unique to plants and fungi (Verrier et al. 2008; Crouzet et al. 2006). Two of the better studied WBC members in Arabidopsis are known to be involved in cuticular lipid transport: AtABCG11/COF1/DSO/WBC11 (At1g17840) and AtABCG12/CER5/AtWBC12 (At1g51500) (Pighin et al. 2004; Bird et al. 2007). A third WBC member has a known role in kanamycin resistance, apparently by sequestration of the kanamycin itself, AtABCG19/AtWBC19 (At3g55130) (Mentewab and Stewart 2005). In comparison, PDR subgroup transporters are proposed to be involved in both abiotic and biotic stress responses AtABCG40/PDR12 (At1g15520) (Crouzet et al. 2006) and resistance to xenobiotics AtABCG37/PDR9 (At3g53480) (Ito and Gray 2006). It is expected that other ABCG subfamily members in plants have diverse roles as the subfamily has greatly expanded to have 43 members in Arabidopsis (Verrier

et al. 2008), compared to less than ten members in vertebrates (Dean and Annilo 2005), where they are known for sterol transport and multidrug resistance (MDR). Of the ABCG proteins in Arabidopsis, three are predicted to be localized to the plastid: AtABCG7/WBC7 (At2g01320), AtABCG23/WBC23 (At5g19410), and AtABCG35/PDR7 (At1g15210), see Table 1 and Fig. 1.

AtABCG7 and AtABCG23 are both members of the WBC subgroup that have not been specifically studied. Their functions are unknown as no obvious phenotype is seen in Arabidopsis T-DNA insertion lines that abolish gene expression (Ajjawi et al. 2010). *AtABCG7* has some level of expression in most tissues, with leaves and stems having the highest expression and roots having the lowest expression (Hruz et al. 2008). The protein clusters with OsABCG1 and ZmABCG40 when analyzed phylogenetically, likely indicating a conserved function among land plants (Pang et al. 2013). When looking at tissue-specific expression datasets, *AtABCG7* seems to be co-expressed with several genes encoding other plastid proteins including a plastid ribosomal protein, At3g63490 (PCC, 0.89), a fructose-bisphosphate aldolase (FBA1), At2g21330 (PCC, 0.88), and a photosystem component, PSBY At1g67740 (PCC, 0.88) (Hruz et al. 2008), which support its plastidic localization. Unlike *AtABCG7*, *AtABCG23* has very low expression in most tissues included in publically available microarray data sets, with the highest expression being in roots, stems, and hypocotyls. Also unlike *AtABCG7*, *AtABCG23* seems to be coexpressed with genes encoding non-plastidic transporters including a xanthine/uracil permease family protein At1g49960 (PCC, 0.85) and two other ABCG family transporters AtABCG6 (PCC, 0.82) and AtABCG20 (PCC, 0.82) (Hruz et al. 2008). In fact, AtABCG23 is one of the few ABC proteins for which no peptides have been identified in proteomic studies, probably because of its low abundance. Therefore, the lack of coexpression of its gene with genes encoding plastidic proteins could indicate that the predicted targeting information is incorrect. AtABCG23 does seem to have a conserved function among land plants, as it clusters with OsABCG13 and ZmABC36 (Pang et al. 2013). Based on its gene expression in non-green tissues, it is likely that if AtABCG23's conserved function is plastidic, that its function is necessary in non-photosynthetic tissues.

AtABCG35 is a member of the PDR subgroup, and there are proteomic data supporting its localization to both the plastid and the plasma membrane (Table 1). AtABCG35 phylogenetically clusters with AtABCG36/PDR8/PEN3 (At1g59870) and AtABCG29/PDR1 (At3g16340) with which it shares 85 % or 71 % identity, respectively (Larkin et al. 2007a). It then forms a small clade with ZmABCG4, ZmABCG8, ZmABCG1, and OsABCG42 (Pang et al. 2013), indicating that gene differentiations in this subgroup probably occurred recently. Both *AtABCG35* and *AtABCG36* have some level of expression in most tissues with the highest expression levels in either roots, *AtABCG35*, or green tissues, *AtABCG36*. These two genes also respond similarly to different stresses such as upregulation in response to cyclohexamide and cold/dark treatments and downregulation in response to abscisic acid and salt treatments (van den Brule and Smart 2002), all of which corroborate a recent gene duplication event. AtABCG36 has been confirmed to be involved in resistance to pathogens which enter by direct penetration, likely by

transporting a fungal toxin (Stein et al. 2006). With the many similarities between AtABCG35 and AtABCG36, it may be expected that AtABCG35 has a similar function in resistance against biotic stress, but it is unclear how a similar activity would be of use in the plastid. *AtABCG35* shares significant coexpression with several genes encoding non-plastid proteins such as a mismatch specific endonuclease, At4g21600 (PCC 0.90), an AT rich DNA-binding protein, At2g45430 (PCC 0.90), and a scopolin beta-glucosidase At1g66270 (PCC 0.89), but none of this information helps to further elucidate its function (Hruz et al. 2008).

11 AtABCI10 and AtABCI12

In this chapter we have already discussed in detail a number of the ABCI family members, which are similar to the multigene ABC transporters. However, there are two from which peptides have been identified in plastidic proteomic databases that have yet to be discussed: AtABCI10/NAP13 (At4g33460) and AtABCI12/MIL23.15 (At3g21580), see Table 1. Their function in plastids is unknown, and they have not been specifically studied.

AtABCI10 is an NBD encoding gene of unknown function. The protein may be associated with additional domains to make a functional transporter, or it may have a function unassociated with transport, thus in Fig. 1, it is shown in the stroma as its associations are unknown. The gene is more highly expressed in shoot than root tissues with no exceptional expression peaks (Hruz et al. 2008). It is coexpressed with At1g69390 (PCC 0.76) which encodes a plastid protein involved in chloroplast replication and At2g42130 (PCC 0.74) which encodes a gene with unknown function but which has a plastid lipid associated protein (PAP) domain. These coexpressed genes are consistent with the proteomics results indicating a plastid localization of AtABCI10. Phenotypes of Arabidopsis with T-DNA insertions that disrupt gene function of *AtABCI10* have not been reported. The gene is conserved among land plants, forming a small clade with ZmABC16 and OsABCI6 (Pang et al. 2013), implying a conserved, although unknown, function.

AtABCI12 has recently been assigned a new family designation as an "Energy-Coupling Factor Transporter" or ECF (Neubauer et al. 2009). Related proteins have three components, including two NBDs from the ABC transporter family and its own transmembrane component which can be distinguished from those of the ABC transporter family (Rodionov et al. 2009). AtABCI12 represents a transmembrane component from the ECF family and seems to be the only representative of this family in Arabidopsis. In spite of its singularity, T-DNA insertions in *AtABCI12* that disrupt gene function do not have strong growth phenotypes in Arabidopsis (Ajjawi et al. 2010), implying that AtABCI12 is either not essential under standard growth chamber conditions or that it has functional redundancy with another family of transporters. *AtABCI12* seems to be expressed most highly in sperm cells which may indicate a function outside of plastids, with medium expression in shoot tissues, and low expression in root tissues (Hruz et al. 2008). It is most closely

coexpressed with At5g62840 (PCC 0.76) a gene encoding a plastid phosphoglycerate mutase family protein and At1g14030 (PCC 0.75) encoding lysine methyltransferase which methylates plastidic fructose 1,6-bisphosphate aldolases. These coexpression data suggest a role for AtABCI12 related to plastidic carbon usage. As an alternative substrate hypothesis, the ECF family has been studied in association with transition metal and heme transport, and AtABCI12 could transport metal ions or coordinated ions. Again, no data can yet confirm or deny these hypotheses, and the function of AtABCI12 remains unknown, although of interest due to its uniqueness.

12 Summary: Roles of ABC Proteins in Plastids

In this chapter, we have considered the various types of ABC proteins that are plastid localized either based on proteomics, prediction or both. At least two conclusions of interest can be drawn from the array of ABC proteins in the plastid. First, in spite of its prokaryotic origin, the plastid contains many representatives of the "eukaryotic" families of ABC proteins, including ABCB, ABCC, ABCD, ABCF, and ABCG. This finding is in line with a recent review of the host origin of all plastid transporters, which confirmed that eukaryotic inheritance is a common theme among multiple transporter families (Tyra et al. 2007). Second, our understanding of transport by ABC proteins in the plastid is severely lacking in spite of knowing molecules that require transport in the plastid and are therefore potential ABC transporter substrates (Rolland et al. 2012). It seems likely that many of the ABC transporters respond to stress conditions as Arabidopsis mutants do not have strong phenotypes under standard conditions (*abcf2*, *abcf5*, *abcb14*, *abcb15*, *abcb26*, *abcb28*, *abcd2*, *abci12*, *abcg7*, and *abcg35*). Others are the major transporter of a specific metabolite and have very strong phenotypes when mutated in Arabidopsis (*sufb*, *sufc*, *sufd*, *tgd1*, *tgd2*, *tgd3*, *abci11*, and *abci22*). Surprisingly, not even all of those with strong phenotypes have been well studied. Our current understanding implies that ABC proteins in the plastid have essential roles in Fe-S cluster formation (SUFB, C, and D), lipid transport (TGD1, 2, and 3), and possibly iron transport (AtABCI11, AtABCI22, and AtABCB29). Additional roles may include, but cannot be limited to, IAA transport into or out of the plastid (AtABCB14 and 15), plastid transcription (ABCF2 and 5), pathogen resistance (AtABCC12) and cadmium resistance (AtABCB29). At this time multiple ABC proteins also exist with completely unknown functions (AtABCB26, AtABCB28, AtABCD2, AtABCG7, AtABCG23, AtABCG35, AtABCI10, AtABCI12, and AtABCI8). Future work on ABC proteins, particularly on those with unidentified functions, is likely to match more of the predicted substrates of plastid transporters with their transporter.

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References

Ajjawi I, Lu Y, Savage LJ, Bell SM, Last RL (2010) Large-scale reverse genetics in Arabidopsis: case studies from the Chloroplast 2010 Project. Plant Physiol 152(2):529–540. doi:10.1104/pp.109.148494

Aloni R, Schwalm K, Langhans M, Ullrich CI (2003) Gradual shifts in sites of free-auxin production during leaf-primordium development and their role in vascular differentiation and leaf morphogenesis in Arabidopsis. Planta 216(5):841–853. doi:10.1007/s00425-002-0937-8

Andersson M (2013) Chloroplast contact to the endoplasmic reticulum and lipid trafficking. In: Biswal B, Krupinska K, Biswal UC (eds) Plastid development in leaves during growth and senescence, vol 36, Advances in photosynthesis and respiration. Springer, Netherlands, pp 155–167. doi:10.1007/978-94-007-5724-0_8

Arabidopsis Genome I (2000) Analysis of the genome sequence of the flowering plant Arabidopsis thaliana. Nature 408(6814):796–815. doi:10.1038/35048692

Awai K, Xu C, Tamot B, Benning C (2006) A phosphatidic acid-binding protein of the chloroplast inner envelope membrane involved in lipid trafficking. Proc Natl Acad Sci U S A 103:10817–10822

Badri DV, Quintana N, El Kassis EG, Kim HK, Choi YH, Sugiyama A, Verpoorte R, Martinoia E, Manter DK, Vivanco JM (2009) An ABC transporter mutation alters root exudation of phytochemicals that provoke an overhaul of natural soil microbiota. Plant Physiol 151(4):2006–2017. doi:10.1104/pp. 109.147462

Badri DV, Chaparro JM, Manter DK, Martinoia E, Vivanco JM (2012) Influence of ATP-binding cassette transporters in root exudation of phytoalexins, signals, and in disease resistance. Front Plant Sci 3:149. doi:10.3389/fpls.2012.00149

Baker A, Graham IA, Holdsworth M, Smith SM, Theodoulou FL (2006) Chewing the fat: beta-oxidation in signalling and development. Trends Plant Sci 11(3):124–132. doi:10.1016/j.tplants.2006.01.005

Balk J, Pilon M (2011) Ancient and essential: the assembly of iron-sulfur clusters in plants. Trends Plant Sci 16(4):218–226. doi:10.1016/j.tplants.2010.12.006

Bayer RG, Stael S, Csaszar E, Teige M (2011) Mining the soluble chloroplast proteome by affinity chromatography. Proteomics 11(7):1287–1299. doi:10.1002/pmic.201000495

Bernard DG, Cheng Y, Zhao Y, Balk J (2009) An allelic mutant series of ATM3 reveals its key role in the biogenesis of cytosolic iron-sulfur proteins in Arabidopsis. Plant Physiol 151(2):590–602. doi:10.1104/pp. 109.143651

Bird D, Beisson F, Brigham A, Shin J, Greer S, Jetter R, Kunst L, Wu XW, Yephremov A, Samuels L (2007) Characterization of Arabidopsis ABCG11/WBC11, an ATP binding cassette (ABC) transporter that is required for cuticular lipid secretion. Plant J 52(3):485–498. doi:10.1111/j.1365-313X.2007.03252.x

Block M, Dubots E, Maréchal E (2013) Glycerolipid biosynthesis and chloroplast biogenesis. In: Biswal B, Krupinska K, Biswal UC (eds) Plastid development in leaves during growth and senescence, vol 36, Advances in photosynthesis and respiration. Springer, Netherlands, pp 131–154. doi:10.1007/978-94-007-5724-0_7

Bouige P, Laurent D, Piloyan L, Dassa E (2002) Phylogenetic and functional classification of ATP-binding cassette (ABC) systems. Curr Protein Pept Sci 3(5):541–559. doi:10.2174/1389203023380486

Brautigam A, Weber APM (2009) Proteomic analysis of the proplastid envelope membrane provides novel insights into small molecule and protein transport across proplastid membranes. Mol Plant 2(6):1247–1261. doi:10.1093/Mp/Ssp070

Breckels LM, Gatto L, Christoforou A, Groen AJ, Lilley KS, Trotter MWB (2013) The effect of organelle discovery upon sub-cellular protein localisation. J Proteom 88:129–140. doi:10.1016/j.jprot.2013.02.019

Chahal HK, Outten FW (2012) Separate FeS scaffold and carrier functions for SufB(2)C(2) and SufA during in vitro maturation of [2Fe2S] Fdx. J Inorgan Biochem 116:126–134. doi:10.1016/j.jinorgbio.2012.06.008

Chen S, Sanchez-Fernandez R, Lyver ER, Dancis A, Rea PA (2007) Functional characterization of AtATM1, AtATM2, and AtATM3, a subfamily of Arabidopsis half-molecule ATP-binding cassette transporters implicated in iron homeostasis. J Biol Chem 282(29):21561–21571. doi:10.1074/jbc.M702383200

Coleman JA, Quazi F, Molday RS (2013) Mammalian P4-ATPases and ABC transporters and their role in phospholipid transport. Biochimica et biophysica acta 1831(3):555–574. doi:10.1016/j.bbalip.2012.10.006

Couturier J, Touraine B, Briat JF, Gaymard F, Rouhier N (2013) The iron-sulfur cluster assembly machineries in plants: current knowledge and open questions. Front Plant Sci 4:259. doi:10.3389/fpls.2013.00259

Crouzet J, Trombik T, Fraysse AS, Boutry M (2006) Organization and function of the plant pleiotropic drug resistance ABC transporter family. FEBS Lett 580(4):1123–1130. doi:10.1016/j.febslet.2005.12.043

Davies P (2010) Regulatory factors in hormone action: level, location and signal transduction. In: Davies P (ed) Plant hormones. Springer, Netherlands, pp 16–35. doi:10.1007/978-1-4020-2686-7_2

Dean M, Annilo T (2005) Evolution of the ATP-binding cassette (ABC) transporter superfamily in vertebrates. Annu Rev Genom Hum G 6:123–142. doi:10.1146/annurev.genom.6.080604.162122

Dubots E, Botte C, Boudiere L, Yamaryo-Botte Y, Jouhet J, Marechal E, Block MA (2012) Role of phosphatidic acid in plant galactolipid synthesis. Biochimie 94(1):86–93. doi:10.1016/j.biochi.2011.03.012

Ferro M, Brugiere S, Salvi D, Seigneurin-Berny D, Court M, Moyet L, Ramus C, Miras S, Mellal M, Le Gall S, Kieffer-Jaquinod S, Bruley C, Garin J, Joyard J, Masselon C, Rolland N (2010) AT_CHLORO, a comprehensive chloroplast proteome database with subplastidial localization and curated information on envelope proteins. Mol Cell Proteom 9(6):1063–1084. doi:10.1074/mcp.M900325-MCP200

Garcia O, Bouige P, Forestier C, Dassa E (2004) Inventory and comparative analysis of rice and Arabidopsis ATP-binding cassette (ABC) systems. J Mol Biol 343(1):249–265. doi:10.1016/j.jmb.2004.07.093

Giroud C, Gerber A, Eichenberger W (1988) Lipids of Chlamydomonas reinhardtii. Analysis of molecular species and intracellular site(s) of biosynthesis. Plant Cell Physiol 29:587–595

Gray MW (2012) Mitochondrial evolution. Cold Spring Harb Perspect Biol 4(9):a011403. doi:10.1101/cshperspect.a011403

Guskov A, Kern J, Gabdulkhakov A, Broser M, Zouni A, Saenger W (2009) Cyanobacterial photosystem II at 2.9-angstrom resolution and the role of quinones, lipids, channels and chloride. Nat Struct Mol Biol 16(3):334–342. doi:10.1038/Nsmb.1559

Heinz E, Roughan G (1983) Similarities and differences in lipid metabolism of chloroplasts isolated from 18:3 and 16:3 plants. Plant Physiol 72:273–279

Higgins CF (1992) ABC transporters - from microorganisms to man. Annu Rev Cell Biol 8:67–113. doi:10.1146/annurev.cb.08.110192.000435

Higgins CF, Linton KJ (2003) ABC transporters: An introduction and overview. In: Holland IB, Susan PCC, Karl K, Christopher F. HigginsA2 - I. Barry Holland SPCCKK, Christopher FH (eds) ABC Proteins. Academic, London, pp xvii–xxiii. 10.1016/B978-012352551-2/50001-9

Hinnebusch AG (1997) Translational regulation of yeast GCN4 - a window on factors that control initiator-tRNA binding to the ribosome. J Biol Chem 272(35):21661–21664. doi:10.1074/jbc.272.35.21661

Hjorth E, Hadfi K, Zauner S, Maier UG (2005) Unique genetic compartmentalization of the SUF system in cryptophytes and characterization of a SufD mutant in Arabidopsis thaliana. FEBS Lett 579(5):1129–1135. doi:10.1016/j.febslet.2004.12.084

Holland IB (2011) ABC transporters, mechanisms and biology: an overview. Essays Biochem 50:1–17. doi:10.1042/Bse0500001

Hollenstein K, Dawson RJ, Locher KP (2007) Structure and mechanism of ABC transporter proteins. Curr Opin Struct Biol 17(4):412–418. doi:10.1016/j.sbi.2007.07.003

Hruz T, Laule O, Szabo G, Wessendorp F, Bleuler S, Oertle L, Widmayer P, Gruissem W, Zimmermann P (2008) Genevestigator v3: a reference expression database for the meta-analysis of transcriptomes. Adv Bioinform 2008:420747. doi:10.1155/2008/420747

Huang CF, Yamaji N, Mitani N, Yano M, Nagamura Y, Ma JF (2009) A bacterial-type ABC transporter is involved in aluminum tolerance in rice. Plant Cell 21(2):655–667. doi:10.1105/tpc.108.064543

Huang CF, Yamaji N, Ma JF (2010) Knockout of a bacterial-type ATP-binding cassette transporter gene, AtSTAR1, results in increased aluminum sensitivity in Arabidopsis. Plant Physiol 153 (4):1669–1677. doi:10.1104/pp. 110.155028

Ito H, Gray WM (2006) A gain-of-function mutation in the Arabidopsis pleiotropic drug resistance transporter PDR9 confers resistance to auxinic herbicides. Plant Physiol 142(1):63–74. doi:10.1104/pp. 106.084533

Jaquinod M, Villiers F, Kieffer-Jaquinod S, Hugouvieux V, Bruley C, Garin J, Bourguignon J (2007) A proteomics dissection of Arabidopsis thaliana vacuoles isolated from cell culture. MolCell Proteom 6(3):394–412

Joo SH, Chung HS, Raetz CR, Garrett TA (2012) Activity and crystal structure of Arabidopsis thaliana UDP-N-acetylglucosamine acyltransferase. Biochemistry 51(21):4322–4330. doi:10.1021/bi3002242

Joshi HJ, Hirsch-Hoffmann M, Baerenfaller K, Gruissem W, Baginsky S, Schmidt R, Schulze WX, Sun Q, van Wijk KJ, Egelhofer V, Wienkoop S, Weckwerth W, Bruley C, Rolland N, Toyoda T, Nakagami H, Jones AM, Briggs SP, Castleden I, Tanz SK, Millar AH, Heazlewood JL (2011) MASCP Gator: an aggregation portal for the visualization of Arabidopsis proteomics data. Plant Physiol 155(1):259–270. doi:10.1104/pp. 110.168195

Jouhet J, Marechal E, Block MA (2007) Glycerolipid transfer for the building of membranes in plant cells. Progr Lipid Res 46(1):37–55. doi:10.1016/j.plipres.2006.06.002

Kamimoto Y, Hamamoto M, Shitan N, Yazaki K (2009) Unusual expression of an Arabidopsis ATP-binding cassette transporter ABCC11. Plant Biotechnol 26 (2):261-265. doi:DOI 10.5511/plantbiotechnology.26.261

Kaneda M, Schuetz M, Lin BSP, Chanis C, Hamberger B, Western TL, Ehlting J, Samuels AL (2011) ABC transporters coordinately expressed during lignification of Arabidopsis stems include a set of ABCBs associated with auxin transport. J Exp Bot 62(6):2063–2077. doi:10.1093/Jxb/Erq416

Kang J, Park J, Choi H, Burla B, Kretzschmar T, Lee Y, Martinoia E (2011) Plant ABC transporters. The Arabidopsis Book 9:e0153. doi:10.1199/tab.0153

Kato T, Tabata S, Sato S (2009) Analyses of expression and phenotypes of knockout lines for Arabidopsis ABCF subfamily members. Plant Biotechnol 26(4):409–414. doi:10.5511/plantbiotechnology.26.409

Katoh H, Hagino N, Grossman AR, Ogawa T (2001) Genes essential to iron transport in the cyanobacterium Synechocystis sp. strain PCC 6803. J Bacteriol 183(9):2779–2784. doi:10.1128/JB.183.9.2779-2784.2001

Kaundal R, Saini R, Zhao PX (2010) Combining machine learning and homology-based approaches to accurately predict subcellular localization in Arabidopsis. Plant Physiol 154 (1):36–54. doi:10.1104/pp. 110.156851

Keeling PJ (2010) The endosymbiotic origin, diversification and fate of plastids. Philos Trans R Soc B 365(1541):729–748. doi:10.1098/rstb.2009.0103

Klein M, Geisler M, Suh SJ, Kolukisaoglu HU, Azevedo L, Plaza S, Curtis MD, Richter A, Weder B, Schulz B, Martinoia E (2004) Disruption of AtMRP4, a guard cell plasma membrane ABCC-type ABC transporter, leads to deregulation of stomatal opening and increased drought susceptibility. Plant J 39(2):219–236. doi:10.1111/j.1365-313X.2004.02125.x

Kobae Y, Sekino T, Yoshioka H, Nakagawa T, Martinoia E, Maeshima M (2006) Loss of AtPDR8, a plasma membrane ABC transporter of Arabidopsis thaliana, causes hypersensitive cell death upon pathogen infection. Plant and Cell Physiol 47(3):309–318. doi:10.1093/Pcp/Pcj001

Kougioumoutzi E, Cartolano M, Canales C, Dupre M, Bramsiepe J, Vlad D, Rast M, Dello Ioio R, Tattersall A, Schnittger A, Hay A, Tsiantis M (2013) SIMPLE LEAF3 encodes a ribosome-associated protein required for leaflet development in Cardamine hirsuta. Plant J 73(4):533–545. doi:10.1111/Tpj.12072

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23(21):2947–2948

Larsen PB, Cancel J, Rounds M, Ochoa V (2007) Arabidopsis ALS1 encodes a root tip and stele localized half type ABC transporter required for root growth in an aluminum toxic environment. Planta 225(6):1447–1458. doi:10.1007/s00425-006-0452-4

Layer G, Gaddam SA, Ayala-Castro CN, Choudens SO, Lascoux D, Fontecave M, Outten FW (2007) SufE transfers sulfur from SufS to SufB for iron-sulfur cluster assembly. J Biol Chem 282(18):13342–13350. doi:10.1074/jbc.M608555200

Li C, Guan Z, Liu D, Raetz CR (2011) Pathway for lipid A biosynthesis in Arabidopsis thaliana resembling that of Escherichia coli. Proc Natl Acad Sci U S A 108(28):11387–11392. doi:10.1073/pnas.1108840108

Liang X, Qin L, Liu P, Wang M, Ye H (2013) Genes for iron-sulfur cluster assembly are targets of abiotic stress in rice, Oryza sativa. Plant Cell Environ. doi:10.1111/pce.12198

Linton KJ, Higgins CF (1998) The Escherichia coli ATP-binding cassette (ABC) proteins. Mol Microbiol 28(1):5–13

Locher KP (2009) Review. Structure and mechanism of ATP-binding cassette transporters. Philos Trans R Soc Lond B, Biol Sci 364(1514):239–245. doi:10.1098/rstb.2008.0125

Lu B, Benning C (2009) A 25-amino acid sequence of the Arabidopsis TGD2 protein is sufficient for specific binding of phosphatidic acid. J Biol Chem 284(26):17420–17427

Lu YP, Li ZS, Drozdowicz YM, Hortensteiner S, Martinoia E, Rea PA (1998) AtMRP2, an Arabidopsis ATP binding cassette transporter able to transport glutathione S-conjugates and chlorophyll catabolites: functional comparisons with AtMRP1. Plant Cell 10(2):267–282. doi:10.1105/Tpc.10.2.267

Lu B, Xu C, Awai K, Jones AD, Benning C (2007) A small ATPase protein of Arabidopsis, TGD3, involved in chloroplast lipid import. J Biol Chem 282:35945–35953

Malinverni JC, Silhavy TJ (2009) An ABC transport system that maintains lipid asymmetry in the gram-negative outer membrane. Proc Natl Acad Sci USA 106(19):8009–8014. doi:10.1073/pnas.0903229106

Marin E, Divol F, Bechtold N, Vavasseur A, Nussaurne L, Forestier C (2006) Molecular characterization of three Arabidopsis soluble ABC proteins which expression is induced by sugars. Plant Sci 171(1):84–90. doi:10.1016/j.plantsci.2006.02.014

Marton MJ, deAldana CRV, Qiu HF, Chakraburtty K, Hinnebusch AG (1997) Evidence that GCN1 and GCN20, translational regulators of GCN4, function on elongating ribosomes in activation of eIF2 alpha kinase GCN2. Mol Cell Biol 17(8):4474–4489

Mentewab A, Stewart CN Jr (2005) Overexpression of an Arabidopsis thaliana ABC transporter confers kanamycin resistance to transgenic plants. Nat Biotechnol 23(9):1177–1180. doi:10.1038/nbt1134

Moller SG, Kunkel T, Chua NH (2001) A plastidic ABC protein involved in intercompartmental communication of light signaling. Genes Dev 15(1):90–103

Nachin L, Loiseau L, Expert D, Barras F (2003) SufC: an unorthodox cytoplasmic ABC/ATPase required for [Fe-S] biogenesis under oxidative stress. EMBO J 22(3):427–437. doi:10.1093/emboj/cdg061

Nagane T, Tanaka A, Tanaka R (2010) Involvement of AtNAP1 in the regulation of chlorophyll degradation in Arabidopsis thaliana. Planta 231(4):939–949. doi:10.1007/s00425-010-1099-8

Neubauer O, Alfandega A, Schoknecht J, Sternberg U, Pohlmann A, Eitinger T (2009) Two essential arginine residues in the T components of energy-coupling factor transporters. J Bacteriol 191(21):6482–6488. doi:10.1128/JB.00965-09

Obayashi T, Kinoshita K (2009) Rank of correlation coefficient as a comparable measure for biological significance of gene coexpression. DNA Res 16(5):249–260. doi:10.1093/dnares/dsp016

Pang KY, Li YJ, Liu MH, Meng ZD, Yu YL (2013) Inventory and general analysis of the ATP-binding cassette (ABC) gene superfamily in maize (Zea mays L.). Gene 526(2):411–428. doi:10.1016/j.gene.2013.05.051

Pighin JA, Zheng HQ, Balakshin LJ, Goodman IP, Western TL, Jetter R, Kunst L, Samuels AL (2004) Plant cuticular lipid export requires an ABC transporter. Science 306(5696):702–704. doi:10.1126/science.1102331

Puig S, Penarrubia L (2009) Placing metal micronutrients in context: transport and distribution in plants. Curr Opin Plant Biol 12(3):299–306. doi:10.1016/j.pbi.2009.04.008

Rayapuram N, Hagenmuller J, Grienenberger JM, Giege P, Bonnard G (2007) AtCCMA interacts with AtCcmB to form a novel mitochondrial ABC transporter involved in cytochrome c maturation in Arabidopsis. J Biol Chem 282(29):21015–21023. doi:10.1074/jbc.M704091200

Rees DC, Johnson E, Lewinson O (2009) ABC transporters: the power to change. Nat Rev Mol Cell Biol 10(3):218–227. doi:10.1038/nrm2646

Reyes-Prieto A, Weber AP, Bhattacharya D (2007) The origin and establishment of the plastid in algae and plants. Annu Rev Genet 41:147–168. doi:10.1146/annurev.genet.41.110306.130134

Rodionov DA, Hebbeln P, Eudes A, ter Beek J, Rodionova IA, Erkens GB, Slotboom DJ, Gelfand MS, Osterman AL, Hanson AD, Eitinger T (2009) A novel class of modular transporters for vitamins in prokaryotes. J Bacteriol 191(1):42–51. doi:10.1128/JB.01208-08

Rolland N, Curien G, Finazzi G, Kuntz M, Marechal E, Matringe M, Ravanel S, Seigneurin-Berny D (2012) The biosynthetic capacities of the plastids and integration between cytoplasmic and chloroplast processes. Annu Rev Genet 46:233–264. doi:10.1146/annurev-genet-110410-132544

Roston R, Gao J, Xu C, Benning C (2011) Arabidopsis chloroplast lipid transport protein TGD2 disrupts membranes and is part of a large complex. Plant J 66(5):759–769. doi:10.1111/j.1365-313X.2011.04536.x

Roston RL, Gao JP, Murcha MW, Whelan J, Benning C (2012) TGD1,-2, and-3 proteins involved in lipid trafficking form ATP-binding cassette (ABC) transporter with multiple substrate-binding proteins. J Biol Chem 287(25):21406–21415. doi:10.1074/jbc.M112.370213

Saini A, Mapolelo DT, Chahal HK, Johnson MK, Outten FW (2010) SufD and SufC ATPase activity are required for iron acquisition during in vivo Fe-S cluster formation on SufB. Biochemistry 49(43):9402–9412. doi:10.1021/bi1011546

Sanchez-Fernandez R, Davies TGE, Coleman JOD, Rea PA (2001) The Arabidopsis thaliana ABC protein superfamily, a complete inventory. J Biol Chem 276(32):30231–30244. doi:10.1074/jbc.M103104200

Sandberg G, Gardestrom P, Sitbon F, Olsson O (1990) Presence of indole-3-acetic-acid in chloroplasts of Nicotiana-Tabacum and Pinus-Sylvestris. Planta 180(4):562–568. doi:10.1007/Bf02411455

Saurin W, Hofnung M, Dassa E (1999) Getting in or out: early segregation between importers and exporters in the evolution of ATP-binding cassette (ABC) transporters. J Mol Evol 48(1):22–41. doi:10.1007/Pl00006442

Seigneurin-Berny D, Gravot A, Auroy P, Mazard C, Kraut A, Finazzi G, Grunwald D, Rappaport F, Vavasseur A, Joyard J, Richaud P, Rolland N (2006) HMA1, a new

Cu-ATPase of the chloroplast envelope, is essential for growth under adverse light conditions. J Biol Chem 281(5):2882–2892. doi:10.1074/jbc.M508333200
Shimoni-Shor E, Hassidim M, Yuval-Naeh N, Keren N (2010) Disruption of Nap14, a plastid-localized non-intrinsic ABC protein in Arabidopsis thaliana results in the over-accumulation of transition metals and in aberrant chloroplast structures. Plant Cell Environ 33(6):1029–1038. doi:10.1111/j.1365-3040.2010.02124.x
Shingles R, North M, McCarty RE (2002) Ferrous ion transport across chloroplast inner envelope membranes. Plant Physiol 128(3):1022–1030. doi:10.1104/pp. 010858
Simm S, Papasotiriou DG, Ibrahim M, Leisegang MS, Muller B, Schorge T, Karas M, Mirus O, Sommer MS, Schleiff E (2013) Defining the core proteome of the chloroplast envelope membranes. Front Plant Sci 4:11. doi:10.3389/fpls.2013.00011
Stein M, Dittgen J, Sanchez-Rodriguez C, Hou BH, Molina A, Schulze-Lefert P, Lipka V, Somerville S (2006) Arabidopsis PEN3/PDR8, an ATP binding cassette transporter, contributes to nonhost resistance to inappropriate pathogens that enter by direct penetration. Plant Cell 18(3):731–746. doi:10.1105/tpc.105.038372
Stevanovic M, Hahn A, Nicolaisen K, Mirus O, Schleiff E (2012) The components of the putative iron transport system in the cyanobacterium Anabaena sp. PCC 7120. Environ Microbiol 14 (7):1655–1670. doi:10.1111/j.1462-2920.2011.02619.x
Sudre D (2009) Identification of membrane proteins involved in heavy metal detoxification and transport in Arabidopsis thaliana. Diss Univ Zèurich, 2009 - Ref Enrico Martinoia, Zèurich
Sun Q, Zybailov B, Majeran W, Friso G, Olinares PDB, van Wijk KJ (2009) PPDB, the plant proteomics database at Cornell. Nucl Acids Res 37:D969–D974. doi:10.1093/Nar/Gkn654
Tameshige T, Fujita H, Watanabe K, Toyokura K, Kondo M, Tatematsu K, Matsumoto N, Tsugeki R, Kawaguchi M, Nishimura M, Okada K (2013) Pattern dynamics in adaxial-abaxial specific gene expression are modulated by a plastid retrograde signal during Arabidopsis thaliana leaf development. Plos Genet 9(7):e1003655. doi:10.1371/journal.pgen.1003655
Tanz SK, Castleden I, Hooper CM, Vacher M, Small I, Millar HA (2013) SUBA3: a database for integrating experimentation and prediction to define the SUBcellular location of proteins in Arabidopsis. Nucleic Acids Res 41(Database issue):D1185–D1191. doi:10.1093/nar/gks1151
Tyra HM, Linka M, Weber AP, Bhattacharya D (2007) Host origin of plastid solute transporters in the first photosynthetic eukaryotes. Genome Biol 8(10):R212. doi:10.1186/Gb-2007-8-10-R212
Tyzack JK, Wang XM, Belsham GJ, Proud CG (2000) ABC50 interacts with eukaryotic initiation factor 2 and associates with the ribosome in an ATP-dependent manner. J Biol Chem 275 (44):34131–34139. doi:10.1074/jbc.M002868200
van den Brule S, Smart CC (2002) The plant PDR family of ABC transporters. Planta 216(1):95–106. doi:10.1007/s00425-002-0889-z
van der Heide T, Poolman B (2002) ABC transporters: one, two or four extracytoplasmic substrate-binding sites? EMBO Rep 3(10):938–943. doi:10.1093/embo-reports/kvt201
van Wijk KJ, Baginsky S (2011) Plastid proteomics in higher plants: current state and future goals. Plant Physiol 155(4):1578–1588. doi:10.1104/pp. 111.172932
Verrier PJ, Bird D, Burla B, Dassa E, Forestier C, Geisler M, Klein M, Kolukisaoglu U, Lee Y, Martinoia E, Murphy A, Rea PA, Samuels L, Schulz B, Spalding EJ, Yazaki K, Theodoulou FL (2008) Plant ABC proteins–a unified nomenclature and updated inventory. Trends Plant Sci 13 (4):151–159. doi:10.1016/j.tplants.2008.02.001
Wang Z, Benning C (2012) Chloroplast lipid synthesis and lipid trafficking through ER-plastid membrane contact sites. Biochem Soc Trans 40:457–463. doi:10.1042/Bst20110752
Wanke D, Kolukisaoglu HU (2010) An update on the ABCC transporter family in plants: many genes, many proteins, but how many functions? Plant Biol 12:15–25. doi:10.1111/j.1438-8677.2010.00380.x
Weber APM, Linka N (2011) Connecting the plastid: transporters of the plastid envelope and their role in linking plastidial with cytosolic metabolism. Annu Rev Plant Biol 62:53–77. doi:10.1146/annurev-arplant-042110-103903

Wohlbach DJ, Quirino BF, Sussman MR (2008) Analysis of the Arabidopsis histidine kinase ATHK1 reveals a connection between vegetative osmotic stress sensing and seed maturation. Plant Cell 20(4):1101–1117. doi:10.1105/tpc.107.055871

Wojas S, Hennig J, Plaza S, Geisler M, Siemianowski O, Sklodowska A, Ruszczynska A, Bulska E, Antosiewicz DM (2009) Ectopic expression of Arabidopsis ABC transporter MRP7 modifies cadmium root-to-shoot transport and accumulation. Environ Pollut 157 (10):2781–2789. doi:10.1016/j.envpol.2009.04.024

Wollers S, Layer G, Garcia-Serres R, Signor L, Clemancey M, Latour JM, Fontecave M, Ollagnier de Choudens S (2010) Iron-sulfur (Fe-S) cluster assembly: the SufBCD complex is a new type of Fe-S scaffold with a flavin redox cofactor. J Biol Chem 285(30):23331–23341. doi:10.1074/jbc.M110.127449

Xin XF, Nomura K, Underwood W, He SY (2013) Induction and suppression of PEN3 focal accumulation during Pseudomonas syringae pv. tomato DC3000 infection of Arabidopsis. Mol Plant Microbe Interact 26(8):861–867. doi:10.1094/Mpmi-11-12-0262-R

Xing U, Jie QIU, Yang XU, Chen-wu XU (2012) Molecular evolution and expression analysis of subfamily ABCB transporter genes in rice. Chn J Rice Sci 26(2):127–136. doi:10.3969/j.issn.1001ª27216.2012.02.001

Xu XM, Moller SG (2004) AtNAP7 is a plastidic SufC-like ATP-binding cassette/ATPase essential for Arabidopsis embryogenesis. Proc Natl Acad Sci USA 101(24):9143–9148. doi:10.1073/pnas.0400799101

Xu C, Fan J, Riekhof W, Froehlich JE, Benning C (2003) A permease-like protein involved in ER to thylakoid lipid transfer in Arabidopsis. EMBO J 22(10):2370–2379

Xu C, Fan J, Froehlich J, Awai K, Benning C (2005a) Mutation of the TGD1 chloroplast envelope protein affects phosphatidate metabolism in Arabidopsis. Plant Cell 17:3094–3110

Xu XM, Adams S, Chua NH, Moller SG (2005b) AtNAP1 represents an atypical SufB protein in Arabidopsis plastids. J Biol Chem 280(8):6648–6654. doi:10.1074/jbc.M413082200

Yang H, Murphy AS (2009) Functional expression and characterization of Arabidopsis ABCB, AUX 1 and PIN auxin transporters in Schizosaccharomyces pombe. Plant J 59(1):179–191. doi:10.1111/j.1365-313X.2009.03856.x

Yu HS, Hu SY, Zhu C (1989) Ultrastructure of sperm cells and the male germ unit in pollen tubes of Nicotiana-Tabacum. Protoplasma 152(1):29–36. doi:10.1007/Bf01354237

Zeng WQ, Brutus A, Kremer JM, Withers JC, Gao XL, Jones AD, He SY (2011) A genetic screen reveals Arabidopsis stomatal and/or apoplastic defenses against Pseudomonas syringae pv. tomato DC3000. Plos Pathog 7(10):e1002291

ABCG Transporters and Their Role in the Biotic Stress Response

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Abstract Pleiotropic drug resistance (PDR) transporters belong to subfamily G of the ATP-Binding Cassette (ABC) family. Plant species contain approximately 15–20 *PDR* genes, the majority of which are still uncharacterized. However, several *PDR* genes have been studied in more detail and it appears that most of them are involved in the plant response to biotic as well as abiotic stress. In this chapter we focus on those *PDR* genes that have shown to play a role in the plant resistance to various pathogens. Characterization of *PDR* knockout or silenced mutants clearly showed the major implication of those genes in the plant defense. However the molecular function of the encoded transporters is still elusive because their substrates have been identified only in a few cases. Another question that needs to be resolved is whether plant PDRs are able, like some fungal or mammalian ABC transporters, to translocate several structurally unrelated substrates.

1 Introduction

The natural environment provides living organisms with all essentialities, but the environment also contains various kinds of stresses. For plants, water or nutrient depletion as well as toxic compound accumulation are forms of abiotic stress resulting in complex responses such as on sensory, transcriptional, and posttranscriptional levels enabling these sessile organisms to adapt to their environment. Biotic stress is imposed by other living organisms and might considerably harm the performance of the plant. The response of the plant to this type of stress is equally complex and includes hormonal responses, transcriptional regulation of gene

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expression, posttranslational modifications of proteins, protein trafficking, and so forth (Kumar 2013; Glombitza et al. 2004; Huang et al. 2011; Peer 2011; Atkinson and Urwin 2012; Kosová et al. 2011; Sunkar 2010). Cells are the basic structural and functional units of all known living organisms. They are separated from their external environment by a membrane barrier, which enables the controlled translocation of molecules and metabolites between the inside and outside of the cell in order to respond to various kinds of stresses. Different transport systems reside in this membrane allowing exchanges on the cellular level and also between the organism and its environment. One of the largest protein families known is the ATP-binding cassette (ABC) family which is present in all known living organisms from bacteria to humans (Henikoff et al. 1997; Kos and Ford 2009). The ABC family comprises soluble and membrane-bound proteins, most of the latter being transporters (Rea 2007). All members of the ABC family share a cytosolic domain known as the ATP-binding cassette or the nucleotide-binding domain (NBD). Besides NBDs, membrane-bound ABC transporters possess transmembrane domains (TMDs), which are composed of several hydrophobic helices and which are less conserved than NBDs. ABC transporters are minimally constituted of two NBDs and two TMDs: the two NBDs supply the transport process with energy by ATP binding and hydrolysis, whereas the two TMDs form the region involved in substrate recognition and translocation through the membrane (Higgins 1992). The ABC family consists of several subfamilies which differ in their structural organization. In contrast to other subfamilies, subfamily G is characterized by reverse oriented motifs (NBD-TMD) and contains both half-size proteins, known as WBC (white–brown complex) and full-size proteins, known as PDR (pleiotropic drug resistance) transporters. Full-size ABCG/PDR transporters have to date only been found in plants, fungi, oomycetes, brown algae, and slime molds (Kang et al. 2011).

In plants, a large number of full-size ABCG transporters have been identified and organized into five distinct clusters (Crouzet et al. 2006; Fig. 1). SpABCG1/SpTUR2 from *Spirodela polyrrhiza* was the first plant ABCG transporter to be identified (Smart and Fleming 1996) and was later suggested to be involved in the biotic stress response (van den Brûle et al. 2002). Although several ABCG isoforms have been linked to the response to biotic and/or abiotic stress by now, the majority of the isoforms have not been fully characterized yet (Table 1). Here we summarize the advances made in identifying the expression and function of ABCG transporters in relation to the complex response mechanisms of plants to biotic stress.

2 Two *Nicotiana* Isoforms, *NpPDR1* and *NtPDR1*, Are Involved in Pathogen Defense

Plants possess a large number of ABC transporters in comparison to other organisms, such as mammals Rea (2007). These transporters have been found to function in various physiological processes, playing for example a role in the extrusion of metabolites before their concentration reaches cellular toxic levels. Looking at the

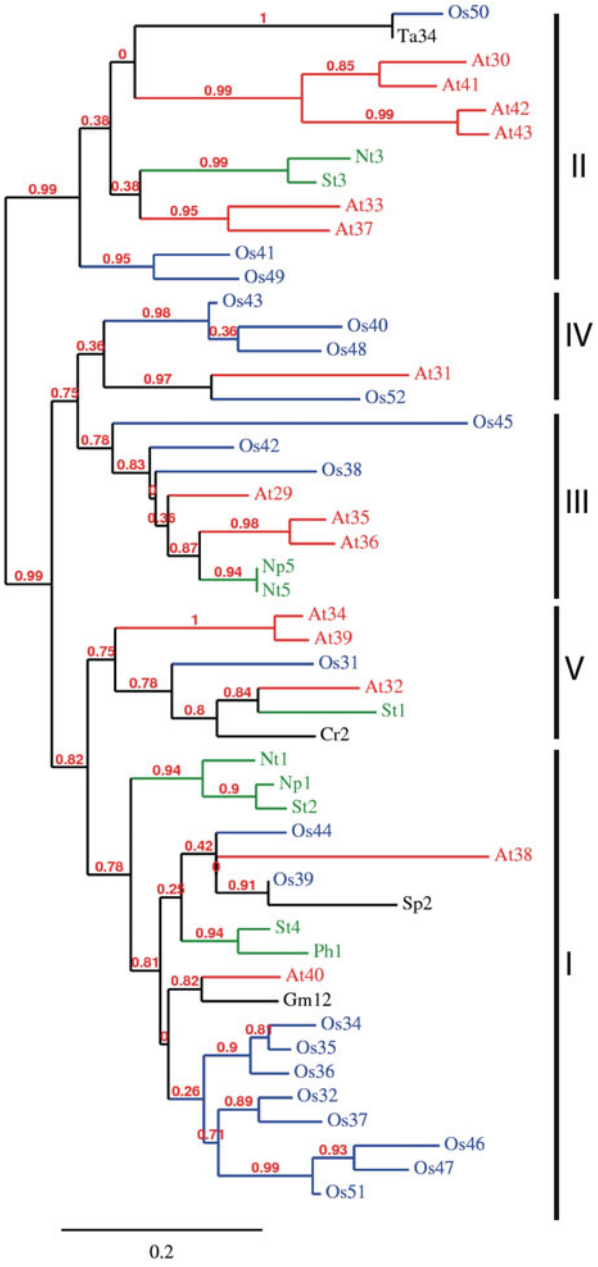

Fig. 1 Phylogenetic analysis of plant ABCG/PDR sequences. Full-length protein sequences of all *Arabidopsis thaliana* and *Oriza sativa* PDR transporters as well as of PDR transporters from other species mentioned in this chapter were aligned (T.Coffee) and a phylogenetic tree determined (Maximum Likelihood) using Phylogeny.fr (www.phylogeny.fr; Dereeper et al., 2008) with 100 bootstrap replicates. The five clades numbered I–V are as in Crouzet et al. (2006). The *scale bar* indicates the number of amino acid substitution per site. Accession numbers of

high number of secondary metabolites that are synthesized upon the recognition of biotic stress, it falls into place that ABC transporters are players in the complex mechanism of plant defense. The first identified ABC transporter which was linked to the biotic stress response is NpPDR1, formerly named NpABC1, which is an ABCG/PDR transporter from *Nicotiana plumbaginifolia* (Jasinski et al. 2001). This protein was identified under the assumption that ABC transporters are involved in the secretion of metabolites under biotic stress conditions and that their expression is regulated by various interrelated metabolic changes. In that particular study, plants were treated with sclareolide, a close analog of the antifungal diterpene sclareol which is produced at the leaf surface of *Nicotiana* species. The expression of a 160 kD protein was induced under these conditions and was found to encode a full-size ABCG transporter, NpPDR1 (Jasinski et al. 2001). Its expression was shown to not only be induced by sclareolide but also by sclareol in *N. plumbaginifolia* leaves and in cell cultures, and it was found to be localized to the plasma membrane of the cell (Jasinski et al. 2001; Grec et al. 2003; Stukkens et al. 2005). Along with the induction of NpPDR1 expression in cell cultures, the cells acquired the ability to excrete radiolabeled sclareolide, supporting the hypothesis that NpPDR1 is an active transporter of sclareol/sclareolide (Jasinski et al. 2001). Sclareol is synthesized in the trichomes of *Nicotiana* ssp. (Guo and Wagner 1995), which is also where NpPDR1 is expressed (Stukkens et al. 2005). Secretion onto the leaf surface via the trichomes matches the distribution of sclareol under biotic stress conditions, where it is believed to act as a protectant against fungal invaders (Bailey et al. 1975; Stukkens et al. 2005).

As sclareol is considered to be a defense related secondary metabolite, the influence of plant hormones that are related to the biotic stress response on NpPDR1 expression was tested. Whereas treatment with salicylic acid (SA) and ethylene did not result in modulated NpPDR1 expression, treatment with methyl jasmonate (MeJA) led to an increase in protein abundance (Grec et al. 2003). As a plant hormone, MeJA initiates diverse metabolic processes in plants in response to developmental or external signals. MeJA has been shown to induce diterpene synthesis (Keinänen et al. 2001), and it has also been shown to function as a signal molecule playing a role in plant defense, particularly during herbivory and infestation by necrotrophic pathogens (Schaller and Stintzi 2008). Infection of *N. plumbaginifolia* by *Botrytis cinerea*, a necrotrophic fungus, induced the expression of NpPDR1 in most leaf tissues, revealing a link between induced plant defense and NpPDR1 protein abundance (Stukkens et al. 2005). Most importantly,

Fig. 1 (continued) Arabidopsis thaliana (Aa, *red*) and *Oriza sativa* (Os, *blue*) full-size ABCG (PDR) are given in Verrier et al. (2008). Solanaceae PDR are in *green*: *Nicotiana tabacum* (Nt) PDR1 (Q76CU2), PDR3 (CAH39853), PDR5 (AFN42936); *Nicotiana plumbaginifolia* (Np) PDR1 (Q949G3); *Solanum tuberosum* (St) PDR1 (AY165018), PDR2 (AY165019), PDR3 (AY165020), PDR4 (AY165021); *Petunia hybrida* (Ph) PDR1; PDR from other species are in *black*: *Catharanthus roseus* (Cr) TPT2 (KC511771); *Glycine max* (Gm) PDR2 (CAK03587); *Triticum aestivum* (Ta) Lr34 (ADK62371); *Spirodela polyrhiza* (Sp) TUR2 (Z70524)

Table 1 Main properties of characterized plant ABCG/PDR transporters

Protein[a]	Abiotic stress	Biotic stress	Hormones and others	Phenotype	Subcellular localization	Tissue expression	Substrate[b]	References
AtABCG30 AtPDR2				Root exudates		Root	*Unidentified root exudates*	Badri et al. (2009)
AtABCG31 AtPDR3				Guard cell response to ABA		Guard cells	*ABA?*	Galbiati et al. (2008)
AtABCG32 AtPDR4				Cuticule structure	PM	Epidermis of expanding organs of above-ground tissues	*Cutin precursors?*	Bessire et al. (2011)
AtABCG33 AtPDR5			Red light					Molas et al. (2006)
AtABCG36 AtPDR8	Cadmium Drought Salt	Bacteria Fungi	SA	Resistance to pathogens, cadmium, IBA, 2,4-DB, drought, and salt	PM	Leaf epidermis Root hairs Root epidermis	*IBA?* *Cadmium?*	Kobae et al. (2006), Stein et al. (2006), Kim et al. (2007), Xin et al. (2013), Strader and Bartel (2009), Strader and Bartel (2011)
AtABCG37 AtPDR9	Iron deficiency		SA Herbicides NPA	Resistance to 2,4-D, NPA, and IBA	PM	Root epidermis	2,4-D IBA Phenolics	Strader and Bartel (2011), Ruzicka et al. (2010), Ito and Gray (2006), Fourcroy et al. (2014)
AtABCG39 AtPDR11	Paraquat, salt, drought, cadmium, H_2O_2			Paraquat tolerance	PM			Xi et al. (2012)
AtABCG40 AtPDR12	Lead	Fungi	SA MeJA Sclareol Ethylene Red light	Resistance to sclareol, drought , and lead		Broad expression Guard cells	ABA	Molas et al. (2006),Campbell et al. (2003), Lee et al. (2005), Kang et al. (2010)
NtPDR1		Bacterial and fugal elicitors	MeJA		PM	Leaf Root Flower	Sclareol	Sasabe et al. (2002), Crouzet et al. (2013)

(continued)

Table 1 (continued)

Protein[a]	Abiotic stress	Biotic stress	Hormones and others	Phenotype	Subcellular localization	Tissue expression	Substrate[b]	References
NpABCG1 NpPDR1		Fungi Oomycetes	JA Sclareol Sclareolide Abietic acid Larixol Cembrane	Resistance to sclareol and pathogens	PM	Leaf Root Flower	Sclareol	Jasinski et al. (2001), Stukkens et al. (2005), Bultreys et al. (2009), Grec et al. (2003)
NpABCG2 NpPDR2						Root Flower Style		Trombik et al. (2008)
NtPDR3	Iron deficiency		NAA SA MeJA Cembrene		PM			Ducos et al. (2005)
NtABCG5 NtPDR5	Wounding	Herbivory Fungi	MeJA	Resistance to *Manduca sexta*	PM	Root Stem Flower Leaf		Bienert et al. (2012)
CrTPT2			MeJA			Epidermis of young leaves	Catharanthine	Yu and De Luca (2013)
OsABCG36 OsPDR9	Hypoxic stress, Heavy metals, Redox dis-tur-bance, Weak organic acids, Salt stress PEG	*Striga hermontica*, *Magnaporthe grisea*	JA NAA IAA BAP			Root Callus Leaf		Moons (2003), Moons (2008), Swarbrick et al. (2008)
OsABCG44 OsPDR17		*Striga hermontica*						Moons (2008), Swarbrick et al. (2008)
OsABCG51 OsPDR20	DTT Lactic acid Malic acid Citric acid GSSG PEG		JA SA ABA IAA			Root Leaf		Moons (2003), Moons (2008)

StABCG1 StPDR1	Salt	*Botrytis cinerea*, *Phytophthora infestans*	Sclareol DAS			Root Tuber Stem Leaf		Ruocco et al. (2011)
StABCG2 StPDR2	Salt	*Phytophthora infestans*, *Botrytis cinerea*	Sclareol 2,4-D SM			Root Leaf		Ruocco et al. (2011)
StABCG3 StPDR3	Salt	*Phytophthora infestans*	DMSO A-solanine			Root		Ruocco et al. (2011)
StABCG4 StPDR4	Salt	*Phytophhthora infestans*	DMSO 2,4-D			Root Tuber Stem Leaf		Ruocco et al. (2011)
GmABCG1 GmPDR12		*Pseudo-monas syringae*	SA MeJA					Eichhorn et al. (2006)
SpABCG1 SpTUR2	Salt stress Cold stress Rhodamine		ABA Sclareol Cyclo-heximide	Resistance to sclareol	PM		Sclareol	Smart and Fleming (1996), van den Brûle et al. (2002)
PaABCG1 PaPDR1					PM	Root	GR24	Kretzschmar et al. (2012)
PgPDR1			SA Chilling ABA MeJA			Root Leaf		Zhang et al. (2013a)
PgPDR3			MeJA			Root		Zhang et al. (2013b)
PhABCG1 PhPDR1	Phosphate starvation	Arbuscular mycor-rhizal colonization	GR24 NAA	Less arbuscular mycorrhizal col-onization Less strigolactone in root exudates		Root	Strigolactone	Kretzschmar et al. (2012)
TaABCG1 TaPDR1	Aluminium Calcium	*Fusarium graminearum*	DON					Shang et al. (2009)

(continued)

Table 1 (continued)

Protein[a]	Abiotic stress	Biotic stress	Hormones and others	Phenotype	Subcellular localization	Tissue expression	Substrate[b]	References
TaLr34				Resistance to leaf rust, stripe rust, stem rust, powdery mildew, Stimulation of senescence-like processes		Flag leaf of mature plants	*Antifungal compound?*	Krattinger et al. (2009) and references therein
HvABCG31 HvPDR6				Leaf water loss, Cutin dis-organization		Young leaves		Chen et al. (2011)

[a]Abbreviations: *Arabidopsis thaliana* (At); *Nicotiana tabacum* (Nt); *Nicotiana plumbaginifolia* (Np); *Catharanthus roseus* (Cr); *Oryza sativa* (Os); *Solanum tuberosum* (St); *Glycine max* (Gm); *Spirodela polyrrhiza* (Sp); *Petunia axillaris* (Pa); *Panax ginseng* (Pg); *Petunia hybrida* (Ph); *Triticum aestivum* (Ta); *Hordeum vulgare* (Hv); *PEG* polyethylene glycol, *ABA* abscisic acid, *JA* jasmonic acid, *MeJA* methyl jasmonate, *SA* salicylic acid, *NAA* 1-naphtalene acetic acid, *BAP* 6-benzylaminopurin, *IAA* indole-3-acetic acid, *DAS* 4,15-diacetoxyscirpenol, *SM* sulfometuron methyl, *IBA* indole-3-butyric acid, *2,4-D* 2,4-dichlorophenoxyacetic acid, *DON* deoxynivalenol, *PM* plasma membrane

[b]A question mark indicates that the substrate has not yet been identified by transport assays

silencing of *NpPDR1* by RNAi markedly increased the sensitivity of plants to *B. cinerea* infection, providing the first demonstration of the role of an ABC transporter in the biotic stress response (Stukkens et al. 2005). A possible explanation for this is that an antimicrobial secondary metabolite, such as sclareol, is excreted by NpPDR1 on the leaf surface whereas its export is limited in the *NpPDR1*-silenced lines. Interestingly, *NpPDR1*-silenced plants were spontaneously infected by pathogens present in the greenhouse when grown under nonsterile conditions, which occasionally also led to plant death, while the performance of wild-type plants did not vary (Stukkens et al. 2005).

It is noteworthy that NpPDR1 expression is not only responsive to a single diterpene but also shows induction after treatment with abietic acid and larixol, two diterpenes that are structurally related to sclareol (Grec et al. 2003). Although the original hypothesis was that expression of the transporter is induced by the presence of its substrate (Jasinski et al. 2001), another possibility is that NpPDR1 expression belongs to a general stress response, since the diterpenes that are added to the medium are toxic for plant cells at certain concentrations. Though the substrate spectrum of NpPDR1 is not completely known to date, it is believed that sclareol and/or a structurally related diterpene is secreted onto the surface of *N. plumbaginifolia* leaves, thereby protecting the plant from pathogen infection.

In addition to trichomes, NpPDR1 is constitutively expressed in plant roots and petals (Stukkens et al. 2005). Soil-borne pathogens are major elicitors of biotic stress below ground. Most root pathogens are necrotrophic meaning that they kill their host (Okubara and Paulitz 2005). Roots of lines that are silenced in the expression of *NpPDR1* were significantly more sensitive to infections by the fungi *B. cinerea*, *Fusarium oxysporum*, and *Rhizoctonia solani*, and the oomycete pathogen *Phytophtora nicotianae*. In the same manner, petals of *NpPDR1*-silenced plants were more rapidly infected by *B. cinerea* than petals from wild-type *N. plumbaginifolia* plants (Bultreys et al. 2009). This denotes that NpPDR1 plays an important role in the response to pathogen attacks in roots in a similar manner to above ground tissues, most likely by its ability to excrete a certain plant secondary metabolite that is aiding in repelling pathogen invasion or in limiting its development. Whether this metabolite is a diterpene is still unknown. Some ABC transporters have a pleiotropic substrate specificity (Chufan et al. 2013). If this is the case for NpPDR1, it is possible that it transports diterpenes at the leaf level and other substrate/s in roots.

Sharing 84 % amino acid sequence identity, *NtPDR1* is the closest homologue of *NpPDR1* in *N. tabacum* and in *N. plumbaginifolia*. However, they are not orthologues since *NtPDR1* and *NpPDR1* orthologues (>98 % identity) were found in both species. *NtPDR1* was originally identified in culture cells which exhibited an induced expression after treatment with microbial elicitors or MeJA (Sasabe et al. 2002). Supporting the hypothesis that NtPDR1 is involved in the biotic stress response, elements in the transcription promoter, such as a GCC box, W boxes, and JA-responsive elements have been identified (Schenke et al. 2003). Recently, NtPDR1 was investigated in more detail. Its expression profile is similar, at the organ level, to that of NpPDR1, being present in roots, leaves (particularly the

epidermis), and flower organs. However, the expression of NtPDR1 and NpPDR1 differ at the cell type level. Indeed, NtPDR1 is expressed in long trichomes on leaves, stems, and petals (Crouzet et al. 2013). This is different from NpPDR1, whose expression was determined in leaf and stem short trichomes and in the petal epidermis instead of trichomes on flowers (Stukkens et al. 2005).

As mentioned above, NtPDR1 was shown to be induced by certain elicitors or MeJA in culture cells (Sasabe et al. 2002). Confirming the induction of NtPDR1 by MeJA in BY2 suspension cells, Crouzet et al. (2013) identified that the auxin hormone naphthalene acid (NAA) and the diterpenes cembrene, sclareol, and abietic acid also led to an increase in NtPDR1 expression while salicylic acid, abscisic acid, gibberellic acid, the ethylene precursor 1-aminocyclopropane-1-carboxlate, or the secondary metabolites berberine and quercetine did not. To screen for substrates of NtPDR1, the protein was expressed in *N. tabacum* BY2 suspension cells and a range of secondary metabolites were added to the culture medium. Based on the features of ABC transporters which mostly act as exporters, and the fact that NtPDR1 is a plasma membrane-localized protein, the sensitivity of the cells toward a certain metabolite was assayed. Cells overexpressing NtPDR1 were more resistant to the presence of the four diterpenes that were tested (sclareol, manool, abietic acid, and dehydroabietic acid) when compared to the wild-type or control cells, whereas none of the other metabolites that were tested led to a growth difference between the wild-type and NtPDR1 overexpressing cell cultures (Crouzet et al. 2013). Using a radiolabelled analog of the diterpene sclareol, direct transport studies using these culture cells identified that NtPDR1 is truly a transporter of sclareol and it is therefore believed that it plays a role in the plant defense mechanism against biotic threats (Crouzet et al. 2013).

3 NtPDR5 Is Involved in Herbivore Resistance

Herbivores, such as caterpillars, are major threats to plants and trigger plant defense responses upon their attack (Heiling et al. 2010; Onkokesung et al. 2010). Upon caterpillar feeding, the plant activates the synthesis of secondary metabolites which function as chemical defense compounds (Kessler and Baldwin 2001; Steppuhn et al. 2004). Increasing evidence suggests that secondary metabolite secretion into the apoplast, onto the plant surface, or into the rhizosphere is connected to the function of full size ABCG transporters, and it was proposed that these transporters are involved in the secondary metabolite-based response against herbivores (Kretzschmar et al. 2011). Following this hypothesis, an ABCG transporter from *N. tabacum*, NtPDR5, was tested for its involvement in herbivore resistance. The protein is expressed at low levels in leaf tissues under standard growth conditions and is induced after exposure to MeJA, suggesting that NtPDR5 is involved in the biotic stress response (Bienert et al. 2012). Treatment with MeJA leads to numerous responses in plants and interestingly, it also elicits herbivore resistance in many plant species (Baldwin 1998). To test whether NtPDR5 expression not only

responds to treatment with a plant hormone but directly to the presence of a herbivore, larvae of the *N. tabacum* specialist *Manduca sexta* were placed onto *N. tabacum* plants. NtPDR5 expression was strongly induced by *M. sexta* feeding, but only in close proximity to the feeding site (Bienert et al. 2012). It is not clear how this very strong but local expression can be explained, but it might be linked to the physiological role of the protein under these conditions and the substrate that is transported by NtPDR5 in the damaged leaf area. The importance of NtPDR5 expression under *M. sexta* feeding is striking as plants that are silenced in *NtPDR5* expression allow much faster growth and development of the caterpillars compared to larvae feeding on wild-type plants (Bienert et al. 2012).

In *N. attenuata*, a number of secondary metabolites have been shown to be induced upon *M. sexta* feeding (e.g., nicotine or diterpene glycosides) (Steppuhn et al. 2004; Heiling et al. 2010). In *NtPDR5*-silenced plants, none of the major secondary metabolites that are known to function in caterpillar defense were identified to be differentially regulated compared to wild-type plants under caterpillar feeding (Bienert et al. 2012). As the expression is only induced in close proximity to the feeding site it might indicate that NtPDR5 has an important function directly at the wounded leaf area. Potentially, NtPDR5 has a role in deterring continuous feeding, or in the wound repair and healing to limit the damage caused by the caterpillar. Taken together, this means that an important metabolite that most likely represents the substrate of NtPDR5 and which might be an important factor in the defense response of *N. tabacum* to *M. sexta* feeding remains to be identified.

4 An ABCG Transporter of *Catharanthus roseus* Is a Catharanthine Transporter

Recently, an ABCG transporter from *Catharanthus roseus*, CrTPT2, was identified and characterized. It is predominantly expressed in the epidermis of young leaves and functions in the secretion of catharanthine on the leaf surface (Yu and De Luca 2013). The *C. roseus* epidermis of young leaves is an active place where synthesis of many different secondary metabolites is taking place. Amongst those, various monoterpenoid indol alkaloids are being synthesized, one of which is catharanthine whose function is possibly related to herbivore feeding deterrence (Yu and De Luca 2013). Catharanthine accumulates in the wax exudates on the leaf surface where its biological activity might be most potent (Roepke et al. 2010). Interestingly, silencing *CrTPT2* resulted in a decreased catharathine level on the leaf surface, whereas the concentration within the leaf increased by more than ten times (Yu and De Luca 2013; see chapter "Transport of monoterpenoid indole alkaloids in *Catharanthus roseus*").

CrTPT2 clusters phylogenetically with the ABCG transporters *AtABCG32*/*AtPDR4*, *OsABCG31*/*OsPDR6*, and *HvABCG31*/*HvPDR6*, which are known to be

involved in cuticle assembly. *AtABCG32* is believed to export particular cutin precursors from epidermal cells, and a *HvABCG31* mutant exhibited a thin cuticule, suggesting a reduced deposition of cutin on the leaf surface (Bessire et al. 2011; Chen et al. 2011). The cuticle represents an important factor in creating a barrier against pathogen invasion, and its proper composition contributes in defeating pathogen attacks. As suggested for NpPDR1 and NtPDR1, CrTPT2 is an exporter of a secondary metabolite onto the leaf surface, strengthening the idea that certain ABC transporters are indeed important in the deposition of biologically active metabolites onto the leaf surface, which for example aid in the defense against biotic threats. Catharanthine is not only known as an important natural cancer drug but also seems to have a defensive role in plants, as it is toxic to insects and inhibits the growth of fungal spores (Roepke et al. 2010). Similar to *NpPDR1*, *CrTPT2* expression is induced by MeJA (Yu and De Luca 2013) and also the abundance of its substrate, catharanthine, is transcriptionally regulated by the jasmonate-signaling pathway (Zhou et al. 2010) linking *CrTPT2* expression and function to the biotic stress response.

5 ABCG Transporters in *Arabidopsis thaliana* Are Involved in Biotic Stress Responses

Arabidopsis serves as a model plant for which a large number of molecular tools are available. These tools are used to study numerous physiological processes in order to gain insights into complex mechanisms. Genevestigator has been utilized to identify genes involved in the biotic stress response, and more precisely to investigate the transcriptional profile of various ABC transporters in response to jasmonic acid (JA) and SA, two hormones that are well described in relation to the biotic stress response. Hence, several ABCG transporters are upregulated in response to the presence of JA or SA, suggesting that these transporters are linked to pathogen defense and/or the interaction between plants and microorganisms (Kang et al. 2011). Two of them have been characterized in detail and will be described in the following.

6 AtABCG36/AtPDR8 Accumulates at Pathogen Entry Sites and Impacts Pathogen Resistance

AtABCG36/AtPDR8, also called PEN3, was shown to be a player in pathogen defense in a forward genetic screen initiated to identify components in the nonhost resistance of Arabidopsis (Stein et al. 2006). One of the mutants utilized in that study which allowed the increased penetration of the barley powdery mildew *Blumeria graminis f. sp hordei* was identified to carry a mutation in the ABCG transporter AtABCG36/AtPDR8/PEN3 (Stein et al. 2006). AtABCG36/PEN3 is a

plasma membrane-localized transporter and it was speculated that it is involved in either the export of toxic compounds, thereby limiting fungal penetration or the detoxification of fungal toxins. In addition to allowing a higher penetration rate, the *pen3* mutant showed an increased frequency in the formation of elongating secondary hyphae. The decrease in nonhost resistance of the *pen3* mutants was further confirmed using other non-host pathogens, namely *Plectosphaerella cucumerina*, *Erysiphe pisi*, and *Phytophthora infestans*. In all cases, infection of the *pen3* mutant resulted in an increased penetration rate compared to the wild type suggesting that the mutation renders the entry of multiple nonhost pathogens possible (Stein et al. 2006). Surprisingly, *pen3* mutants exhibited increased resistance towards the Arabidopsis powdery mildew which, when infecting *pen3* plants, was associated with an increase in chlorosis and necrosis (Stein et al. 2006). Chlorotic and necrotic lesions on *pen3* mutant plants were also observed after infection with *B. cinerea*, *P. cucumerina*, and *Phakopsora pachyrhizi* (Stein et al. 2006; Loehrer et al. 2008). Genetic crosses with different mutants revealed that AtABCG36/PEN3 functions in a SA-dependent manner and that the SA pathway is hyperactivated in the mutant (Stein et al. 2006). This is a probable explanation for the increased resistance of the plants toward the Arabidopsis powdery mildew infection, as the fungus is an obligate biotroph proliferating on living tissues. In the same year, AtABCG36/PEN3 was shown to elevate the basal plant defense against the bacterial pathogen *Pseudomonas syringae* pv. tomato DC3000 (Kobae et al. 2006). However, this result was recently questioned by Xin et al. (2013) who found that the *pen3* mutant displayed decreased resistance against the same bacterial strain (Xin et al. 2013). Whereas Kobae and coauthors identified the *pen3* mutant to have constitutive elevated expression of defense-related genes, Xin and coauthors did not obtain the same result and highlighted that the increased sensitivity of the *pen3* mutants to abiotic and biotic stresses might under certain growth conditions influence plant performance.

To gain more insights into the function of AtABCG36/PEN3 in relation to fungal and bacterial infections, GFP was fused to AtABCG36/PEN3. The protein was found to be constitutively present and equally distributed in epidermal and mesophyll cells under standard growth conditions (Stein et al. 2006; Xin et al. 2013; Underwood and Somerville 2013). However, upon pathogen infection, PEN3-GFP accumulated at the entry sites of the fungus *B. graminis f. sp hordei* and the bacterium *P. syringae* pv. tomato DC3000 (Stein et al. 2006; Xin et al. 2013). Similar to pathogens, the bacterial elicitor flg22 induced *PEN3* expression and led to a strong focal accumulation of PEN3-GFP, suggesting that AtABCG36/PEN3 accumulates at the bacterial entry site (Xin et al. 2013). This was confirmed, as infection studies with a fluorescence-marker labeled bacterial strain showed that the focal accumulation of PEN3-GFP was associated with the presence of the bacteria (Xin et al. 2013). Flg22 is derived from the bacterial flagellin and is commonly used as an elicitor of pathogen-associated molecular patterns (PAMP). Another PAMP is hydrolyzed chitin, which is associated with the cell walls of fungal pathogens and insects. It was recently shown that in addition to flg22, chitin is recruiting PEN3 to the simulated pathogen detection sites by the perception of the elicitor signal (Underwood and Somerville 2013). In agreement with this, AtABCG36/PEN3 is

not targeted to particular sites in flagellin receptor and chitin receptor mutants in response to treatment with the corresponding elicitor. This implies that specific elicitor receptors are necessary for the focal accumulation of AtABCG36/PEN3 in response to biotic stress (Underwood and Somerville 2013).

In wild-type plants, flg22 treatment leads to a typical defense-response in the form of callose deposition on the cell surface (Clay et al. 2009). Interestingly, these callose rings that are induced by flg22 colocalize to a large extent with the focally accumulated PEN3-GFP protein (Underwood and Somerville 2013). In the *pen3* mutant, callose deposition in response to flg22 treatment is impaired, which supports a positive role of AtABCG36/PEN3 in the resistance to bacterial pathogens (Clay et al. 2009). Furthermore, treatment of *pen3* plants with flg22 prior to infection with *P. syringae* pv. tomato DC3000 increased the susceptibility toward the bacteria compared to wild-type plants in which the priming increased the resistance toward the pathogen (Xin et al. 2013). As the lack of *AtABCG36*/*PEN3* in the mutants resulted in significantly reduced callose deposition upon flg22 treatment compared to the wild type, this might partially explain the lack of priming effect by the elicitor in these plants.

How PEN3 specifically contributes to biotic stress resistance remains mysterious as long as its specific substrate/s in this physiological context is/are not identified. It was speculated that this ABCG transporter exports a fungal toxin or rather specific antimicrobial metabolites (Stein et al. 2006). Identification of this/these metabolite/s will aid in understanding how this transporter is able to impact pathogen resistance and why focal accumulation is initiated upon infection. AtABCG36/PEN3 is also required for callose deposition in response to bacterial elicitor treatment (Clay et al. 2009). Callose deposition might influence pathogen proliferation, but in which manner AtABCG36/PEN3 contributes to this process remains to be identified.

In addition to pathogen resistance, AtABCG36/PEN3 has been associated with heavy metal resistance as a cadmium extrusion pump (Kim et al. 2007) and with the export of the auxin precursor IBA (Strader and Bartel 2009; see chapter "IBA Transport by PDR Proteins"). How cadmium and/or IBA could influence pathogen resistance is not clear. In any case, AtABCG36/PEN3 belongs to the family of the pleitropic drug resistance transporters, some of which are known to transport a large variety of substrates with little common structure in yeast (Rogers et al. 2001). Multiple substrate-binding sites in the cavity of the transporters might explain this feature of this protein family across organisms and might indicate that AtABCG36/PEN3 is a protein that has an impact on multiple physiological processes.

7 AtABC40/AtPDR12 Expression Is Regulated in Response to Biotic Stress Signals

Inoculation by pathogens also impacts the gene expression of other ABC transporters in Arabidopsis. *AtABCG32*/*AtPDR4* has been shown to be downregulated after infection with powdery mildew, whereas *AtABCG40*/*AtPDR12* is upregulated

(Nishimura et al. 2003; Stein et al. 2006). *AtABCG40* was the first ABCG transporter that was shown to be transcriptionally responsive to pathogen infections in Arabidopsis. Inoculation with the incompatible fungal pathogen *Alternaria brassiciola*, the compatible fungal pathogens *Sclerotinia sclerotiorum* and *F. oxysporum*, and the compatible bacterial pathogen *P. syringae* pv. tomato DC3000 resulted in an increase in *AtABCG40* expression, suggesting that this ABC transporter is involved in the biotic stress response (Campbell et al. 2003). However, a knockout mutant in *AtABCG40* did not display any altered susceptibility in response to pathogen inoculation (Campbell et al. 2003), implying that *AtABCG40* does not play a dominant role in the resistance toward the tested pathogens. *AtABCG40* is also transcriptionally regulated by treatment with the antifungal diterpene sclareol (van den Brûle and Smart 2002; Campbell et al. 2003; Seo et al. 2012), and the *AtABCG40* mutant showed a reduced germination rate and reduced root growth on media containing 50 μM sclareol (Campbell et al. 2003). Therefore, *AtABCG40* is likely to be involved in the detoxification of sclareol from the plant which would explain why the mutant is more sensitive to the metabolite than wild-type plants (Campbell et al. 2003). Although Arabidopsis has not been shown to synthesize sclareol endogenously, these findings might indicate that *AtABCG40* is involved in the transport of an antimicrobial secondary metabolite with a secondary structure similar to sclareol.

Recently, sclareol was shown to have an impact on resistance towards the soil-borne bacterial pathogen *Ralstonia solanacearum*. Interestingly, pretreatment of wild-type Arabidopsis plants with sclareol inhibited bacterial growth and this effect was attenuated in *AtABCG40* knockout mutants (Seo et al. 2012). It appears that the induction of AtABCG40 expression by sclareol is important for conferring resistance towards bacterial infection in Arabidopsis. Sclareol and another labdane-type diterpene, *cis*-abienol, were shown to be synthesized in *N. tabacum* upon *R. solanacearum* infection, giving a direct hint that these secondary metabolites play a role in the defense against this pathogen (Seo et al. 2012). Antimicrobials are known to inhibit the growth of fungi as well as bacteria. Seo and coworkers were able to show that treatment with sclareol prior to *R. solanacearum* infection decreased not only bacterial proliferation on Arabidopsis but also on *N. tabacum* and *Solanum tuberosum* plants without having direct antimicrobial activity on the pathogen nor by changing its pathogenicity. Similarly to the *AtABCG40* mutant, silencing of *NtPDR1*, a close homologue of *AtABCG40* in tobacco, attenuated the priming of sclareol which limited the growth of bacteria on wild-type *N. tabacum* plants (Seo et al. 2012). Since sclareol is also toxic for plant cells (Crouzet et al. 2013), one hypothesis is that sclareol toxicity induces a stress response that protects the plant against pathogens.

In addition to sclareol, AtABCG40 has been shown to transport abscisic acid (ABA) (Kang et al. 2010; see chapter "ABA Transport by ABCG Transporter Proteins"). Recently it was suggested, that the induction of *AtABCG40* gene expression by sclareol influences ABA mobility by changing the abundance of the transporter (Seo et al. 2012). Furthermore, the *AtABCG40* knockout mutant has been shown to be more sensitive to lead (Lee et al. 2005). The mutants contained a

higher amount of lead after exposure compared to the wild type and grew slower on lead containing media. By studying the role of glutathione in the ABC transporter mutant background it became clear that *AtABCG40* functions independently of the glutathione detoxification pathway (Lee et al. 2005) and that there has to be an additional mechanism of detoxification. The type of substrate *AtABCG40* is transporting in response to heavy metal stress remains to be elucidated, but it might be yet another secondary metabolite different from ABA and sclareol.

In respect to pathogen resistance, lignin accumulation is a way for the plant to defeat pathogen invasion (Raes et al. 2003). Interestingly, sclareol increased the level of thioglycolic acid lignin, which might indicate that sclareol has a function in respect to this physiological process (Seo et al. 2012). Indeed, an ABCG transporter, AtABCG29/AtPDR1, was shown to transport p-coumaryl alcohol which is a monolignol lignin precursor, across the plasma membrane (Alejandro et al. 2012). It is possible that AtABCG40 might have a similar function in response to biotic stress as AtABCG29. However, whether the different phenotypes of the *AtABCG40* mutant can be related to a common substrate or whether this transporter has a role in different physiological pathways due to its pleiotropic nature remains to be investigated in the future.

8 Wheat PDR Transporters Confer Durable Resistance Against Pathogens

Infections by fungi, bacteria, and viruses are important factors in agricultural management and the development of modern agriculture. The introduction of resistance genes in cultivars that are used for food and feed production is one of the major goals of plant breeding. Many genes have been identified to play a role in the resistance of crop plants to biotic stress. As demonstrated in a transcriptomic study on wheat (*Triticum aestivum*) infected by the barley yellow dwarf virus, a large number of genes are transcriptionally regulated upon infection (Wang et al. 2013). Among these genes, ABCG transporters have been identified to be upregulated and are therefore most likely linked to the biotic stress response.

In respect to pathogen resistance, the quantitative resistance marker *Lr34* has been intensively used in wheat breeding programs for over 100 years without losing its efficiency (Kolmer et al. 2008). When compared to gene–gene resistance this marker functions in the gene-durable resistance against multiple pathogens. *Lr34* has been associated with conferring resistance to leaf rust (*Puccinia triticina*), stripe rust (*P. striiformis*), stem rust (*P. graminis*), and powdery mildew (*Blumeria graminis*), as well as with leaf tip necrosis (Dyck et al. 1966; Dyck 1987, 1991; McIntosh 1992; Singh 1992; Spielmeyer et al. 2005). In addition, it was also found to be genetically associated with resistance to the spot blotch disease (Lillemo et al. 2013). *Lr34* has recently been identified to encode an ABC transporter of the PDR subfamily (Krattinger et al. 2009). The resistant allele of *Lr34* was shown to

harbor two mutations leading to two amino acid substitutions in the first transmembrane domain which connects the two nucleotide-binding domains (Krattinger et al. 2009). This may result in an altered structure, substrate specificity or affinity, protein stability, or activity of the protein (Krattinger et al. 2013).

The expression of Lr34 during leaf tip necrosis is independent of pathogen infection and is most apparent late in plant development. Indeed, a marker gene that is expressed in relation to senescence, *HvS40*, was shown to correlate with leaf tip necrosis and therefore with the expression of *Lr34* (Krattinger et al. 2009). Thus, it is possible that Lr34 plays a role during senescence-like processes, potentially via the export of senescence-related compounds during leaf tip necrosis (Krattinger et al. 2009; Risk et al. 2012).

Introducing the resistant *Lr34* allele (*Lr34res*) in two leaf rust susceptible wheat backgrounds resulted in leaf rust resistant plants comparable to plants carrying the *Lr34* resistant allele endogenously (Risk et al. 2012). Reduced colony growth and smaller infection sites were seen in the susceptible backgrounds carrying the transgene compared to the non-transformed control. In addition, *Lr34*-associated induced leaf tip necrosis was also detected and it was suggested that elevated *Lr34res* expression increases the symptoms of leaf tip necrosis (Risk et al. 2012). Interestingly, these phenotypes were independent of an increased hypersensitive response, altered callose deposition, or increased pathogenesis-related (*PR)* gene expression in the transgenic line, suggesting that Lr34 functions independently of these biotic stress responses. These experiments demonstrate that the expression of the resistant allele carrying this ABCG transporter can be successfully introduced in susceptible wheat varieties and can be utilized to reach the maximal efficiency of this protein in plant breeding (Risk et al. 2012).

Wheat *Lr34* has also been used to test its potential to confer broad-spectrum resistance in barley (*Hordeum vulgare*) (Risk et al. 2013). Barley does not carry an endogenous homolog or ortholog of *Lr34* (Krattinger et al. 2011), making it an interesting grass species to test the functionality of the wheat resistance gene in this organism. The complete genomic sequence of *Lr34res* including the putative promoter sequence and the native terminator was introduced into barley cv. Golden Promise (Risk et al. 2013). Transgenic plants expressing the full-length genomic *Lr34res* sequence showed a strong resistance against barely leaf rust and a reduction in the severity of barley powdery mildew infections. Wheat stem rust is also infectious on barley but grows more slowly. Barley plants expressing wheat *Lr34res* were also infected with this pathogen, but the infection sites failed to grow (Risk et al. 2013). Additionally, expression of wheat *Lr34res* in barley led to elevated *PR* gene expression prior to infection by pathogens and the plants showed symptoms of premature senescence, which is indicative of the leaf tip necrosis phenotype seen in wheat (Risk et al. 2013). Gene expression analysis however showed that the transgene expression throughout the plant growth cycle is comparable to the *Lr34res* expression in transgenic wheat which does not show premature senescence (Risk et al. 2012, 2013). This may indicate posttranscriptional regulations of the protein in wheat that is absent in barley, which may be overcome by using pathogen-inducible or cell type-specific promoters.

In summary, these studies on wheat and barley nicely demonstrate that an ABCG transporter can be used to gain pathogen resistance, not only in the endogenous host species but also in related plant species. The substrate of Lr34 in the context of senescence and pathogen resistance remains to be identified, but it is possible that the pleiotropic features of ABCG transporters and the ability to transport multiple substrates might explain the durable resistance phenotype that is associated with the resistant allele of this gene.

9 Gene Expression of ABCG Transporters Is Regulated Upon Biotic Stress and Hormone or Pathogen Treatment in Various Plant Species

Since ABCG transporters have been associated with the biotic stress response, several studies have shown that gene expression of certain ABCG transporters is regulated in respect thereof. In 2001, an *Oryza sativa* (rice) ABC gene was found to be transcriptionally regulated after infection by a blast fungus (*Pyricularia grisea*) (Xiong et al. 2001). A thorough study on rice ABCG gene expression upon treatment with different elicitors revealed that the response to treatment with JA had an effect on the expression level of most members of this subfamily. In particular, *OsPDR9* was markedly induced by treatment with JA in rice roots (Moons 2003, 2008). In addition, *OsPDR1*, *OsPDR3*, *OsPDR5*, *OsPDR8*, and *OsPDR17* are upregulated in their expression, suggesting that these genes play a role in pathogen defense, wounding response, or the export of secondary metabolites (Moons 2008). In this respect, *OsPDR9* and *OsPDR17* were shown to be transcriptionally upregulated in roots of a resistant rice variety that was infected with the parasitic plant *Striga hermonthica*, while this was not the case for the susceptible rice variety (Swarbrick et al. 2008).

Unlike JA, SA is associated with the local apoptotic hypersensitive response and the establishment of systemic acquired resistance during pathogen attack. This plant hormone greatly induced the expression of *OsPDR20* and to a lesser extent *OsPDR3* (Moons 2008). As the constitutive expression of the ABCG genes in rice roots is not common, it was suggested that ABCG gene expression is induced on demand after a certain stimulus is sensed and the transporter and its function are required.

In *S. tuberosum* (potato), four ABCG isoforms have been cloned and studied for their response to biotic stress elicitors (Ruocco et al. 2011). Cell cultures were used as a model system to investigate the expression patterns of the isoforms following specific treatments. Low expression of *StPDR1* was observed throughout the experiment, and none of the elicitors led to the induction of transcript abundance. However, a decrease in expression was seen after treatment with *B. cinerea* cell walls, 4,15-diacetoxyscirpenol, and sclareol. *StPDR2* exhibited increased expression after treatment with *B. cinerea* cell walls, sclareol, 2,4-dichlorophenoxyacetic

acid, sulfometuron methyl, and sodium chloride. *StPDR3* was constitutively expressed and rather downregulated by most of the added compounds. The expression of *StPDR4* was only slightly influenced throughout the experiments (Ruocco et al. 2011). Most interestingly, infection of potato plants by *P. infenstans* led to the tremendous upregulation of *StPDR1* and *StPDR2* expression in leaves at 48 h post infection, and the timing of induction matched the necrotic phase of infection where the transporters might potentially be important for the transport of a secondary metabolite functional at this stage of the biotic stress response. Infection by *P. infestans* can be divided into different phases; starting with the invasion followed by a biotrophic phase and ending with a necrotic phase. A slight induction of *StPDR3* and *StPDR4* was also observed throughout the experiment. Whereas the *StPDR3* transcript increased consistently over 48 h and then dropped again, expression of *StPDR4* increased for up to 24 h, then decreased again, only to increase once more at 96 h post infection. *StPDR3* might accordingly play a role during the early stages of infection, reaching its maximum at the successful establishment of the necrotic phase, and *StPDR4* might be important at both the early and very late stages of infection. *StPDR2* is the most homologous isoform to *NpPDR1*, and its induction by sclareol makes it the most promising candidate as a functional transporter in the biotic stress response (Ruocco et al. 2011).

The expression of ABCG transporters has also been studied in legumes. The identification of a soybean (*Glycine max*) ABCG transporter (*GmPDR12*) which is induced by SA and MeJA is an indication that these transporters also play a role in pathogen resistance in this plant species. *GmPDR12* was identified in a screen for genes that are induced by SA in soybean culture cells, and its promoter harbors multiple sequences that are known to be important in genes that are involved in the SA response pathway (Eichhorn et al. 2006). Hypothetically, the encoded protein is involved in limiting pathogen infection by the transport of a secondary metabolite that either strengthens the cell wall locally, or defeats pathogen invasion in another way. However, the substrate/s of the transporter remains unknown, and further insights into its specific functions will most certainly be gained by the identification of such.

10 Desired Interactions Between Plants and Microorganisms

Symbiosis between plants and beneficial microorganisms is a common feature seen in nature. Flavonoids are secondary metabolites found in the root exudates of legumes with the aim to attract rhizobia with the ability to fix atmospheric nitrogen and deliver it to the plant. Genistein is one flavonoid found in the root exudate of soybean that functions in the communication between the plant and symbiotic rhizobia. Interestingly, genistein has been suggested to be a substrate of an ABC transporter in soybean roots (Sugiyama et al. 2007, 2008).

ABCG transporters of the half-size family were shown to be major contributors for the establishment of a functional symbiosis between mycorrhiza fungi and *Medicago truncatula* as well as *O. sativa* (Zhang et al. 2010; Gutjahr et al. 2012). Strigolactones, which have been described to be of major importance in the root exudates to promote fungal metabolism and hyphal branching (Akiyama et al. 2005; Besserer et al. 2006), have been speculated to be a substrate of the ABCG transporters STR and STR2 in *M. truncatula* (Zhang et al. 2010), but are most likely not (Gutjahr et al. 2012). However, in *Petunia hybrida* one ABCG transporter, *PDR1*, was shown to be regulated in its expression by colonization of mycorrhiza fungi and treatment with a synthetic strigolactone analogue, GR24 (Kretzschmar et al. 2012). In an analysis of the root exudate of the *PhPDR1* mutants, the strigolactone orobanchol was shown to be present in a lower quantity compared to the wild type. Interestingly, the concentration of the compound in root extracts did not differ from the wild type, suggesting that the secretion of this molecule is impaired in the mutants, that the synthesis is driven to its maximal extent, and that the reduced colonization phenotype of the mutants can be explained by a lack of strigolactone exudation (Kretzschmar et al. 2012).

Additional information on the ABCG transporters that are involved in symbiotic interactions can be found in chapter "Defence, symbiosis and ABCG transporters."

11 Conclusion

While several ABC transporters seem to be involved in transporting toxic secondary metabolites that protect the plant against animals, insect herbivores, and microorganisms, as well as in transporting signaling molecules important in the establishment of symbiosis, it appears that the metabolites which are transported vary according to the transporter and the species, reflecting the large diversity of secondary metabolism in plants. The importance of ABCG transporters in conferring resistance towards fungal, bacterial, and insect pathogens has been shown in different ways ranging from gene expression analysis across various phenotypes, to identifying substrates that function in the biotic stress response. Though the number of characterized ABCG isoforms has increased over the last years (Table 1), the substrate or substrate spectrum of the transporters remains largely unknown, which represents a major bottleneck in understanding the physiological functions of these proteins.

At the leaf level, much progress has been made in understanding the importance of ABC transporters in the complex mechanism of the biotic stress response. Though phenotypes point toward critical functions that are doubtlessly linked to the transport of a plant metabolite to the plant–pathogen interface, little is known about the actual compound that is involved in the process. Advances have been made using cell cultures and applying different secondary metabolites that possibly function in plant defense. However, relatively little success has until now been achieved when studying the metabolite profiles or the exudates of ABC transporter

mutants. It is still unclear whether plant ABC transporters, and PDR transporters in particular, have this astonishing property similar to some animal and fungal ABC transporters to transport a large range of structurally distinct substrates.

The plant root system is hidden below ground, but is extremely important for the communication between plants themselves, plants and soil-borne pathogens, as well as plants and symbiotic partners. ABCG transporters are largely expressed in roots, and here the importance in secreting root exudates has been proven for some Arabidopsis isoforms (Badri et al. 2009). One interesting avenue for further studies would be to shed more light into secretion mechanisms happening below ground that involve ABC transporter-mediated metabolite transport and that are important for the edaphon.

Interestingly, ABC transporters were also identified to play important roles in some plant pathogens. An ABC transporter from *B. cinerea* has for example been shown to be important for the detoxification of the plant fungitoxin camalexin which is secreted in response to abiotic and biotic stress in Arabidopsis (Stefanato et al. 2009). Likewise, mutation of an ABC transporter of *B. cinerea* infecting grapevine results in increased sensitivity to resveratrol, which acts as a plant defense compound (Schoonbeek et al. 2001). This points toward a fine-tuned mechanism to secrete defense or undesired molecules from endogenous cells, involving ABC transporters in each partner of the interaction.

In conclusion, it is extremely fascinating that this transporter family is ubiquitously utilized across organisms of different kingdoms, transporting metabolites to keep the endogenous cells vital, and communicate between cells and between organisms. Expanding the knowledge on the substrate spectrum of the individual transporters will be indispensable to understanding the physiological impact of this protein family and will certainly entail a deeper understanding of the transport mechanisms occurring under biotic stress.

References

Alejandro S, Lee Y, Tohge T, Sudre D, Osorio S, Park J, Bovet L, Lee Y, Geldner N, Fernie AR, Martinoia E (2012) AtABCG29 is a monolignol transporter involved in lignin biosynthesis. Curr Biol 22:1207–1212

Akiyama K, Matsuzaki K, Hayashi H (2005) Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. Nature 435:824–827

Atkinson NJ, Urwin PE (2012) The interaction of plant biotic and abiotic stresses: from genes to the field. J Exp Bot 63:3523–3543

Badri DV, Quintana N, El Kassis EG, Kim HK, Choi YH, Sugiyama A, Verpoorte R, Martinoia E, Manter DK, Vivanco JM (2009) An ABC transporter mutation alters root exudation of phytochemicals that provoke an overhaul of natural soil microbiota. Plant Physiol 151: 2006–2017

Bailey JA, Carter GA, Burden RS, Wain RL (1975) Control of rust diseases by diterpenes from *Nicotiana glutinosa*. Nature 255:328–329

Baldwin IT (1998) Jasmonate-induced responses are costly but benefit plants under attack in native populations. Proc Natl Acad Sci U S A 95:8113–8118

Besserer A, Puech-Pages V, Kiefer P, Gomez-Roldan V, Jauneau A, Roy S, Portais JC, Roux C, Becard G, Sejalon-Delmas N (2006) Strigolactones stimulate arbuscular mycorrhizal fungi by activating mitochondria. PLoS Biol 4:e226

Bessire M, Borel S, Fabre G, Carraça L, Efremova N, Yephremov A, Cao Y, Jetter R, Jacquat AC, Métraux JP, Nawrath C (2011) A member of the PLEIOTROPIC DRUG RESISTANCE family of ATP binding cassette transporters is required for the formation of a functional cuticle in Arabidopsis. Plant Cell 23:1958–1970

Bienert MD, Siegmund SEG, Drozak A, Trombik T, Bultreys A, Baldwin IT, Boutry M (2012) A pleiotropic drug resistance transporter in *Nicotiana tabacum* is involved in defense against the herbivore *Manduca sexta*. Plant J 72:745–757

Bultreys A, Trombik T, Drozak A, Boutry M (2009) *Nicotiana plumbaginifolia* plants silenced for the ATP-binding cassette transporter gene *NpPDR1* show increased susceptibility to a group of fungal and oomycete pathogens. Mol Plant Pathol 10:651–663

Campbell EJ, Schenk PM, Kazan K, Penninckx IA, Anderson JP, Maclean DJ, Cammue BP, Ebert PR, Manners JM (2003) Pathogen-responsive expression of a putative ATP-binding cassette transporter gene conferring resistance to the diterpenoid sclareol is regulated by multiple defense signaling pathways in Arabidopsis. Plant Physiol 133:1272–1284

Chen G, Komatsuda T, Ma JF, Nawrath C, Pourkheirandish M, Tagiri A, Hu YG, Sameri M, Li X, Zhao X, Liu Y, Li C, Ma X, Wang A, Nair S, Wang N, Miyao A, Sakuma S, Yamaji N, Zheng X, Nevo E (2011) An ATP-binding cassette subfamily G full transporter is essential for the retention of leaf water in both wild barley and rice. Proc Natl Acad Sci U S A 108: 12354–12359

Chufan EE, Kapoor K, Sim HM, Singh S, Talele TT, Durell SR, Ambudkar SV (2013) Multiple transport-active binding sites are available for a single substrate on human P-glycoprotein (ABCB1). PLoS One 8:e82463

Clay NK, Adio AM, Denoux C, Jander G, Ausubel FM (2009) Glucosinolate metabolites required for an Arabidopsis innate immune response. Science 323:95–101

Crouzet J, Trombik T, Fraysse AS, Boutry M (2006) Organization and function of the plant pleiotropic drug resistance ABC transporter family. FEBS Lett 580:1123–1130

Crouzet J, Roland J, Peeters E, Trombik T, Ducos E, Nader J, Boutry M (2013) NtPDR1, a plasma membrane ABC transporter from *Nicotiana tabacum*, is involved in diterpene transport. Plant Mol Biol 82:181–192

Dereeper A, Guignon V, Blanc G, Audic S, Buffet S, Chevenet F, Dufayard JF, Guindon S, Lefort V, Lescot M, Claverie JM, Gascuel O (2008) Phylogeny.fr: robust phylogenetic analysis for the non-specialist. Nucleic Acids Res 36(Web Server issue):W465–469

Ducos E, Fraysse AS, Boutry M (2005) NtPDR3, an iron-deficiency inducible ABC transporter in *Nicotiana tabacum*. FEBS Lett 579:6791–6795

Dyck PL, Samborsk DJ, Anderson RG (1966) Inheritance of adult-plant leaf rust resistance derived from common wheat varieties exchange and Frontana. Can J Genet Cytol 8:665–671

Dyck PL (1987) The association of a gene for leaf rust resistance with the chromosome-7D suppressor of stem rust resistance in common wheat. Genome 29:467–469

Dyck PL (1991) Genetics of adult-plant leaf rust resistance in Chinese Spring and sturdy Wheats. Crop Sci 31:309–311

Eichhorn H, Klinghammer M, Becht P, Tenhaken R (2006) Isolation of a novel ABC-transporter gene from soybean induced by salicylic acid. J Exp Bot 57:2193–2201

Fourcroy P, Sisó-Terraza P, Sudre D, Savirón M, Reyt G, Gaymard F, Abadía A, Abadia J, Alvarez-Fernández A, Briat JF (2014) Involvement of the ABCG37 transporter in secretion of scopoletin and derivatives by Arabidopsis roots in response to iron deficiency. New Phytol 201:155–167

Galbiati M, Simoni L, Pavesi G, Cominelli E, Francia P, Vavasseur A, Nelson T, Bevan M, Tonelli C (2008) Gene trap lines identify Arabidopsis genes expressed in stomatal guard cells. Plant J 53:750–762

Glombitza S, Dubuis PH, Thulke O, Welzl G, Bovet L, Götz M, Affenzeller M, Geist B, Hehn A, Asnaghi C, Ernst D, Seidlitz HK, Gundlach H, Mayer KF, Martinoia E, Werck-Reichhart D, Mauch F, Schäffner AR (2004) Crosstalk and differential response to abiotic and biotic stressors reflected at the transcriptional level of effector genes from secondary metabolism. Plant Mol Biol 54:817–835

Grec S, Vanham D, de Ribaucourt JC, Purnelle B, Boutry M (2003) Identification of regulatory sequence elements within the transcription promoter region of *NpABC1*, a gene encoding a plant ABC transporter induced by diterpenes. Plant J 35:237–250

Guo Z, Wagner GJ (1995) Biosynthesis of labdenediol and sclareol in cell-free extracts from trichomes of *Nicotiana glutinosa*. Planta 197:627–632

Gutjahr C, Radovanovic D, Geoffroy J, Zhang Q, Siegler H, Chiapello M, Casieri L, An K, An G, Guiderdoni E, Kumar CS, Sundaresan V, Harrison MJ, Paszkowski U (2012) The half-size ABC transporters STR1 and STR2 are indispensable for mycorrhizal arbuscule formation in rice. Plant J 69:906–920

Heiling S, Schuman MC, Schoettner M, Mukerjee P, Berger B, Schneider B, Jassbi AR, Baldwin IT (2010) Jasmonate and ppHsystemin regulate key Malonylation steps in the biosynthesis of 17-Hydroxygeranyllinalool Diterpene Glycosides, an abundant and effective direct defense against herbivores in *Nicotiana attenuata*. Plant Cell 22:273–292

Henikoff S, Greene EA, Pietrokovski S, Bork P, Attwood TK, Hood L (1997) Gene families: the taxonomy of protein paralogs and chimeras. Science 278:609–614

Higgins CF (1992) ABC transporters: from microorganisms to man. Annu Rev Cell Biol 8:67–113

Huang C, Verrillo F, Renzone G, Arena S, Rocco M, Scaloni A, Marra M (2011) Response to biotic and oxidative stress in Arabidopsis thaliana: analysis of variably phosphorylated proteins. J Proteomics 74:1934–1949

Ito H, Gray WM (2006) A gain-of-function mutation in the Arabidopsis pleiotropic drug resistance transporter PDR9 confers resistance to auxinic herbicides. Plant Physiol 142:63–74

Jasinski M, Stukkens Y, Degand H, Purnelle B, Marchand-Brynaert J, Boutry M (2001) A plant plasma membrane ATP binding cassette-type transporter is involved in antifungal terpenoid secretion. Plant Cell 13:1095–1107

Kang J, Hwang JU, Lee M, Kim YY, Assmann SM, Martinoia E, Lee Y (2010) PDR-type ABC transporter mediates cellular uptake of the phytohormone abscisic acid. Proc Natl Acad Sci U S A 107:2355–2360

Kang J, Park J, Choi H, Burla B, Kretzschmar T, Lee Y, Martinoia E (2011) Plant ABC Transporters. The Arabidopsis book/American Society of Plant Biologists 9:e0153

Keinänen M, Oldham NJ, Baldwin IT (2001) Rapid HPLC screening of jasmonate-induced increases in tobacco alkaloids, phenolics, and diterpene glycosides in *Nicotiana attenuata*. J Agric Food Chem 49:3553–3558

Kessler A, Baldwin IT (2001) Defensive function of herbivore-induced plant volatile emissions in nature. Science 291:2141–2144

Kim DY, Bovet L, Maeshima M, Martinoia E, Lee Y (2007) The ABC transporter AtPDR8 is a cadmium extrusion pump conferring heavy metal resistance. Plant J 50:207–218

Kobae Y, Sekino T, Yoshioka H, Nakagawa T, Martinoia E, Maeshima M (2006) Loss of *AtPDR8*, a plasma membrane ABC transporter of *Arabidopsis thaliana*, causes hypersensitive cell death upon pathogen infection. Plant Cell Physiol 47:309–318

Kolmer JA, Singh RP, Garvin DF, Viccars L, William HM, Huerta-Espino J, Ogbonnaya FC, Raman H, Orford S, Bariana HS, Lagudah ES (2008) Analysis of the *Lr34*/*Yr18* rust resistance region in wheat germplasm. Crop Sci 48:1841–1852

Kos V, Ford RC (2009) The ATP-binding cassette family: a structural perspective. Cell Mol Life Sci 66:3111–3126

Kosová K, Vítámvás P, Prášil IT, Renaut J (2011) Plant proteome changes under abiotic stress–contribution of proteomics studies to understanding plant stress response. J Proteomics 74: 1301–1322

Krattinger SG, Lagudah ES, Spielmeyer W, Singh RP, Huerta-Espino J, McFadden H, Bossolini E, Selter LL, Keller B (2009) A putative ABC transporter confers durable resistance to multiple fungal pathogens in wheat. Science 323:1360–1363

Krattinger SG, Lagudah ES, Wicker T, Risk JM, Ashton AR, Selter LL, Matsumoto T, Keller B (2011) *Lr34* multi-pathogen resistance ABC transporter: molecular analysis of homoeologous and orthologous genes in hexaploid wheat and other grass species. Plant J 65:392–403

Krattinger SG, Jordan DR, Mace ES, Raghavan C, Luo MC, Keller B, Lagudah ES (2013) Recent emergence of the wheat Lr34 multi-pathogen resistance: insights from haplotype analysis in wheat, rice, sorghum and *Aegilops tauschii*. Theor Appl Genet 126:663–72

Kretzschmar T, Burla B, Lee Y, Martinoia E, Nagy R (2011) Functions of ABC transporters in plants. Essays Biochem 50:145–160

Kretzschmar T, Kohlen W, Sasse J, Borghi L, Schlegel M, Bachelier JB, Reinhardt D, Bours R, Bouwmeester HJ, Martinoia E (2012) A petunia ABC protein controls strigolactone-dependent symbiotic signalling and branching. Nature 483:341–344

Kumar PP (2013) Regulation of biotic and abiotic stress responses by plant hormones. Plant Cell Rep 32:943

Lee M, Lee K, Lee J, Noh EW, Lee Y (2005) AtPDR12 contributes to lead resistance in Arabidopsis. Plant Physiol 138:827–836

Lillemo M, Joshi AK, Prasad R, Chand R, Singh RP (2013) QTL for spot blotch resistance in bread wheat line Saar co-locate to the biotrophic disease resistance loci *Lr34* and *Lr46*. Theor Appl Genet 126:711–719

Loehrer M, Langenbach C, Goellner K, Conrath U, Schaffrath U (2008) Characterization of nonhost resistance of Arabidopsis to the Asian soybean rust. Mol Plant Microbe Interact 21:1421–1430

McIntosh RA (1992) Close genetic linkage of genes conferring adult-plant resistance to leaf rust and stripe rust in wheat. Plant Pathol 41:523–527

Molas ML, Kiss JZ, Correll MJ (2006) Gene profiling of the red light signalling pathways in roots. J Exp Bot 57:3217–3229

Moons A (2003) *Ospdr9*, which encodes a PDR-type ABC transporter, is induced by heavy metals, hypoxic stress and redox perturbations in rice roots. FEBS Lett 553:370–376

Moons A (2008) Transcriptional profiling of the PDR gene family in rice roots in response to plant growth regulators, redox perturbations and weak organic acid stresses. Planta 229:53–71

Nishimura MT, Stein M, Hou BH, Vogel JP, Edwards H, Somerville SC (2003) Loss of a callose synthase results in salicylic acid-dependent disease resistance. Science 301:969–972

Okubara PA, Paulitz TC (2005) Root defense responses to fungal pathogens: a molecular perspective. Plant Soil 274:215–226

Onkokesung N, Baldwin IT, Gális I (2010) The role of jasmonic acid and ethylene crosstalk in direct defense of Nicotiana attenuata plants against chewing herbivores. Plant Signal Behav 5: 1305–1307

Peer WA (2011) Plasma membrane protein trafficking. In: Murphy AS, Schulz B, Peer WA (eds) The plant plasma membrane, plant cell monographs, vol 19. Springer, Heidelberg, pp 31–56

Raes J, Rohde A, Christensen JH, Van de Peer Y, Boerjan W (2003) Genome-wide characterization of the lignification toolbox in Arabidopsis. Plant Physiol 133:1051–1071

Rea PA (2007) Plant ATP-binding cassette transporters. Annu Rev Plant Biol 58:347–375

Risk JM, Selter LL, Krattinger SG, Viccars LA, Richardson TM, Buesing G, Herren G, Lagudah ES, Keller B (2012) Functional variability of the *Lr34* durable resistance gene in transgenic wheat. Plant Biotechnol J 10:477–487

Risk JM, Selter LL, Chauhan H, Krattinger SG, Kumlehn J, Hensel G, Viccars LA, Richardson TM, Buesing G, Troller A, Lagudah ES, Keller B (2013) The wheat *Lr34* gene provides resistance against multiple fungal pathogens in barley. Plant Biotechnol J 11:847–854

Roepke J, Salim V, Wu M, Thamm AM, Murata J, Ploss K, Boland W, De Luca V (2010) Vinca drug components accumulate exclusively in leaf exudates of *Madagascar periwinkle*. Proc Natl Acad Sci U S A 107:15287–15292

Rogers B, Decottignies A, Kolaczkowski M, Carvajal E, Balzi E, Goffeau A (2001) The pleiotropic drug ABC transporters from *Saccharomyces cerevisiae*. J Mol Microbiol Biotechnol 3: 207–214

Ruocco M, Ambrosino P, Lanzuise S, Woo SL, Lorito M, Scala F (2011) Four potato () ABCG transporters and their expression in response to abiotic factors and *Phytophthora infestans* infection. J Plant Physiol 168:2225–2233

Ruzicka K, Strader LC, Bailly A, Yang H, Blakeslee J, Langowskia L, Nejedla E, Fujita H, Itoh H, Syono K, Hejatko J, Gray WM, Martinoia E, Geisler M, Bartel B, Murphy AS, Friml J (2010) *Arabidopsis* PIS1 encodes the ABCG37 transporter of auxinic compounds including the auxin precursor indole-3-butyric acid. Proc Natl Acad Sci U S A 107:10749–10753

Sasabe M, Toyoda K, Shiraishi T, Inagaki Y, Ichinose Y (2002) cDNA cloning and characterization of tobacco ABC transporter: *NtPDR1* is a novel elicitor-responsive gene. FEBS Lett 518:164–168

Schaller A, Stintzi A (2008) Jasmonate biosynthesis and signaling for induced plant defense against herbivory. In: Schaller A (ed) Induced plant resistance to herbivory. Springer, Heidelberg, pp 349–366

Shang Y, Xiao J, Ma LL, Wang HY, Qi ZJ, Chen PD, Liu DJ, Wang XE (2009) Characterization of a PDR type ABC transporter gene from wheat (*Triticum aestivum L.*). Chn Sci Bull 54: 3249–3257

Schenke D, Sasabe M, Toyoda K, Inagaki YS, Shiraishi T, Ichinose Y (2003) Genomic structure of the *NtPDR1* gene, harboring the two miniature inverted-repeat transposable elements, *NtToya1* and *NtStowaway101*. Genes Genet Syst 78:409–418

Schoonbeek H, Del Sorbo G, De Waard MA (2001) The ABC transporter BcatrB affects the sensitivity of *Botrytis cinerea* to the phytoalexin resveratrol and the fungicide fenpiclonil. Mol Plant Microbe Interact 14:562–571

Seo S, Gomi K, Kaku H, Abe H, Seto H, Nakatsu S, Neya M, Kobayashi M, Nakaho K, Ichinose Y, Mitsuhara I, Ohashi Y (2012) Identification of natural diterpenes that inhibit bacterial wilt disease in tobacco, tomato and Arabidopsis. Plant Cell Physiol 53:1432–1444

Singh RP (1992) Genetic association of leaf rust resistance gene Lr34 with adult plant resistance to stripe rust in bread wheat. Phytopathology 82:835–838

Smart CC, Fleming AJ (1996) Hormonal and environmental regulation of a plant PDR5-like transporter. J Biol Chem 271:19351–19357

Spielmeyer W, McIntosh RA, Kolmer J, Lagudah ES (2005) Powdery mildew resistance and Lr34/Yr18 genes for durable resistance to leaf and stripe rust cosegregate at a locus on the short arm of chromosome 7D of wheat. Theor Appl Genet 111:731–735

Stefanato FL, Abou-Mansour E, Buchala A, Kretschmer M, Mosbach A, Hahn M, Bochet CG, Métraux JP, Schoonbeek HJ (2009) The ABC transporter BcatrB from *Botrytis cinerea* exports camalexin and is a virulence factor on *Arabidopsis thaliana*. Plant J 58:499–510

Stein M, Dittgen J, Sanchez-Rodriguez C, Hou BH, Molina A, Schulze-Lefert P, Lipka V, Somerville S (2006) Arabidopsis PEN3/PDR8, an ATP binding cassette transporter, contributes to nonhost resistance to inappropriate pathogens that enter by direct penetration. Plant Cell 18:731–746

Steppuhn A, Gase K, Krock B, Halitschke R, Baldwin IT (2004) Nicotine's defensive function in nature. PLoS Biol 2:E217

Strader LC, Bartel B (2009) The Arabidopsis PLEIOTROPIC DRUG RESISTANCE8/ABCG36 ATP binding cassette transporter modulates sensitivity to the auxin precursor indole-3-butyric acid. Plant Cell 21:1992–2007

Strader LC, Bartel B (2011) Transport and metabolism of the endogenous auxin precursor indole-3-butyric acid. Mol Plant 4:477–486

Stukkens Y, Bultreys A, Grec S, Trombik T, Vanham D, Boutry M (2005) NpPDR1, a pleiotropic drug resistance-type ATP-binding cassette transporter from *Nicotiana plumbaginifolia*, plays a major role in plant pathogen defense. Plant Physiol 139:341–352

Sunkar R (2010) MicroRNAs with macro-effects on plant stress responses. Semin Cell Dev Biol 21:805–811

Sugiyama A, Shitan N, Yazaki K (2007) Involvement of a soybean ATP-binding cassette-type transporter in the secretion of genistein, a signal flavonoid in legume-Rhizobium symbiosis. Plant Physiol 144:2000–2008

Sugiyama A, Shitan N, Yazaki K (2008) Signaling from soybean roots to rhizobium: an ATP-binding cassette-type transporter mediates genistein secretion. Plant Signal Behav 3: 38–40

Swarbrick PJ, Huang K, Liu G, Slate J, Press MC, Scholes JD (2008) Global patterns of gene expression in rice cultivars undergoing a susceptible or resistant interaction with the parasitic plant *Striga hermonthica*. New Phytol 179:515–529

Trombik T, Jasinski M, Crouzet J, Boutry M (2008) Identification of a cluster IV pleiotropic drug resistance transporter gene expressed in the style of Nicotiana plumbaginifolia. Plant Mol Biol 66:165–175

Underwood W, Somerville SC (2013) Perception of conserved pathogen elicitors at the plasma membrane leads to relocalization of the Arabidopsis PEN3 transporter. Proc Natl Acad Sci U S A 110:12492–12497

van den Brûle S, Muller A, Fleming AJ, Smart CC (2002) The ABC transporter SpTUR2 confers resistance to the antifungal diterpene sclareol. Plant J 30:649–662

van den Brûle S, Smart CC (2002) The plant PDR family of ABC transporters. Planta 216:95–106

Verrier PJ, Bird D, Burla B, Dassa E, Forestier C, Geisler M, Klein M, Kolukisaoglu Ü, Lee Y, Martinoia E, Murphy A, Rea PA, Samuels L, Schulz B, Spalding EP, Yazaki K, Theodoulou FL (2008) Plant ABC proteins - a unified nomenclature and update inventory. Trends Plant Sci 13:151–159

Wang X, Liu Y, Chen L, Zhao D, Wang X, Zhang Z (2013) Wheat resistome in response to barley yellow dwarf virus infection. Funct Integr Genomics 13:155–165

Xi J, Xu P, Xiang CG (2012) Loss of AtPDR11, a plasma membrane localized ABC transporter, confers paraquat tolerance in *Arabidopsis thaliana*. Plant J 69:782–791

Xin XF, Nomura K, Underwood W, He SY (2013) Induction and suppression of PEN3 focal accumulation during *Pseudomonas syringae* pv. tomato DC3000 infection of Arabidopsis. Mol Plant Microbe Interact 26:861–867

Xiong L, Lee MW, Qi M, Yang Y (2001) Identification of defense-related rice genes by suppression subtractive hybridization and differential screening. Mol Plant Microbe Interact 14: 685–692

Yu F, De Luca V (2013) ATP-binding cassette transporter controls leaf surface secretion of anticancer drug components in *Catharanthus roseus*. Proc Natl Acad Sci U S A 110: 15830–15835

Zhang Q, Blaylock LA, Harrison MJ (2010) Two *Medicago truncatula* half-ABC transporters are essential for arbuscule development in arbuscular mycorrhizal symbiosis. Plant Cell 22: 1483–1497

Zhang R, Zhu J, Cao HZ, An YR, Huang JJ, Chen XH, Mohammed N, Afrin S, Luo ZY (2013a) Molecular cloning and expression analysis of PDR1-like gene in ginseng subjected to salt and cold stresses or hormonal treatment. Plant Physiol Biochem 71:203–211

Zhang R, Huang J, Zhu J, Xie X, Tang Q, Chen X, Luo J, Luo Z (2013b) Isolation and characterization of a novel PDR-type ABC transporter gene PgPDR3 from Panax ginseng C.A. Meyer induced by methyl jasmonate. Mol Biol Rep 40:6195–6204

Zhou ML, Zhu XM, Shao JR, Wu YM, Tang YX (2010) Transcriptional response of the catharanthine biosynthesis pathway to methyl jasmonate/nitric oxide elicitation in *Catharanthus roseus* hairy root culture. Appl Microbiol Biotechnol 88:737–750

Defence, Symbiosis and ABCG Transporters

Joanna Banasiak and Michał Jasiński

Abstract Plant genomes encode more than 100 ATP-binding cassette (ABC) transporters, a number far exceeding those of other organisms. The membrane-bound ABC transporters belonging to the G subfamily (ABCGs) can be categorised by their distinctive topology and taxa distribution. ABCGs form the largest known subfamily of ABC proteins, with 43 and 50 members in *Arabidopsis* and rice, respectively. Collected experimental data have revealed the great functional diversity of these proteins. The substrates known to be transferred by ABCGs, usually through the plasma membrane, include surface lipids, plant hormones and secondary metabolites. Therefore, ABCGs are recognised as being important for plant development as well as interactions with the environment. Historically, certain members of the ABCG subfamily were considered as proteins that evolved to be involved in pathogenic processes or biotic stress responses. However, recent discoveries have demonstrated that the function of ABCGs in plants extends beyond simply the secretion of anti-microbial molecules. Equally important as defence against invaders are interactions of plants with microorganisms that are beneficial to both partners. Such beneficial interactions include (1) symbiotic associations with fungi of the phylum *Glomeromycota,* also known as arbuscular mycorrhizae (AM) and (2) legume–rhizobia symbiosis (LRS). We have only just begun to discover that plant ABC transporters are important modulators of symbioses, but how they participate in these processes is unclear. Here, we provide basic information regarding the members of the G subfamily of ABC transporters and

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position them in the context of the defence reactions and symbiotic associations of plants, with special emphasis on legumes.

1 ABCGs as ABC Transporters

Aligning amino acid sequences and performing phylogenetic analyses have allowed for most eukaryotic ABC proteins to be grouped into eight major subfamilies, which are indicated with a simple, single-letter code (A–H; Verrier et al. 2008). In addition, a subfamily unique to plants, subfamily (I), has been assigned and contains ABCs that are absent from most animal genomes (Verrier et al. 2008). The ABCG proteins share structural features that are common among all ABCs, such as the presence of two characteristic modules, namely, (1) a hydrophobic transmembrane domain (TMD) and (2) a cytosolic region containing an ABC transporter-specific nucleotide-binding domain (NBD). The latter is defined by the presence of a unique amino acid sequence called an ABC signature ([LIVMFY]S[SG]GX3[RKA][LIVLMYA]X[LIVFM][AG]) (Rea 2007).

The building modules of ABCGs are present in the reverse orientation, with the NBD domain approaching the amino terminal end of the protein. The reverse type of topology is characteristic for, markedly expanded in plants, the subfamily G. The expansion of ABCGs in plants appears to have been associated with taxon-specific functional diversification (Verrier et al. 2008). ABCGs fall into two distinct groups: (1) half-size transporters, formerly named the white brown complex (WBC) after the homologues of the prototypical members of this family from *Drosophila melanogaster*, which comprise only one NBD and TMD (NBD–TMD) and (2) full-size transporters (formerly pleiotropic drug resistance transporters, or PDRs), which contain two NBDs and two TMDs $(NBD–TMD)_2$ (Crouzet et al. 2006; Verrier et al. 2008; Kang et al. 2011). The half-size transporters require dimerisation to form a functional ABC transporter, making them structural equivalents of the full-size transporters.

Two NBDs and two TMDs seem necessary to form a functional unit for the translocation of various molecules through biological membranes (McFarlane et al. 2010). Different dimer combinations exist, as predicted from mutant phenotypes, and provide diverse substrates and perform multiple functions (Bird et al. 2007). It is believed that full-size ABCGs originated from WBC gene duplication (Crouzet et al. 2006). Currently, full-size ABCGs have been identified only in plants, fungi, oomycetes, brown algae and slime moulds but not in animals (Anjard et al. 2002; Tyler et al. 2006; Cock et al. 2010). The half-size G proteins have been found more broadly in bacteria and eukaryotes. These proteins share the reverse type of topology with the H subfamily of half-size ABC transporters; however, the latter is unrelated to ABCGs and is absent in plants. The systematic identification and interspecies sequence comparison of plant members of the G

subfamily or solely full-size ABCGs have been conducted in *Arabidopsis thaliana, Oryza sativa, Lotus japonicus* and *Medicago truncatula* (Sugiyama et al. 2006; Verrier et al. 2008; Jasinski et al. 2009).

2 ABCG Substrates and Functional Diversity: The *Arabidopsis* Case

Recent data collected for selected *A. thaliana* ABCGs positioned them as proteins that translocate across biological membranes various molecules, such as abscisic acid (ABA), p-coumaryl alcohol and various lipid precursors. These transport activities have an impact on stomata functioning, lignin composition and cuticle and pollen-wall formation. For example, the lack of the full size AtABCG40, which is a guard cell ABA importer, caused a slower response of the stomata to ABA and reduced drought tolerance (Kang et al. 2010). ABA is also transported by AtABCG25, which is a half-size member of the G subfamily that functions as an ABA exporter in the vascular bundle, where enzymes that biosynthesise this hormone are expressed, and may be responsible for the delivery of ABA to the extracellular environment (Kuromori et al. 2010). Another member of the full-size ABCG subfamily, AtABCG29/PDR1, exhibits a high co-expression ratio with three genes of the phenylpropanoid biosynthesis pathway and an expression pattern in primary stems that is consistent with that of monolignol biosynthetic genes and increased lignin content. Indeed, an analysis of the lignin constituents showed that, in *abcg29* mutants, *p*-hydroxyphenyl, guaiacol and syringyl, as well as a large number of flavonoids and glucosinolates, were present in lower amounts. These results suggested that AtABCG29 is a likely candidate for the transport of phenolic compounds, and heterologous expression using yeasts and plants showed that AtABCG29 is a *p*-coumaryl alcohol exporter (Alejandro et al. 2012).

Phenotype analyses of half-size *atabg11* and *atabg12* mutants revealed reduced amounts of wax and cutin and only waxes in the cuticle, respectively (Bird et al. 2007; Panikashvili et al. 2007). Bimolecular fluorescence complementation (BiFC) assays that accompanied studies on AtABCG11/AtABCG12 demonstrated that these proteins formed heterodimers, and only AtAGCG11 was able to form homodimers (McFarlane et al. 2010). This finding suggests a partner selection during the dimerisation process and possibly functional/substrate specialisation. Finally, loss-of-function mutants of *Arabidopsis* ABCG26 showed a drastic decline in seed production as a consequence of a defect in the transport of sporopollenin precursors and, therefore, pollen maturation (Quilichini et al. 2010; Dou et al. 2011; Choi et al. 2011; see also chapter "Transport of monoterpenoid indole alkaloids in *Catharanthus roseus*"). Noted above, the exemplary activities of the G subfamily of ABC transporters in *Arabidopsis* illustrate their functional/substrate diversity. It is worth to emphasize that the identified transporter(s) represent also considerable biotechnological targets that could potentially improve agricultural traits.

3 Plants and Defence

Plants are repeatedly attacked by a wide range of pathogens that use a variety of strategies to successfully infect the host. Most plants are able to effectively protect themselves against undesirable colonisation. The first line of defence is non-specific and consists of constitutive barriers, both structural (waxy cuticle) and chemical (pre-formed secondary metabolites) (Dangl and Jones 2001). Plants also possess defence mechanisms that are activated during pathogen attack and initiated by the conserved, microbe-specific molecules referred to as microbe- or pathogen-associated molecular patterns (MAMPs/PAMPs), otherwise known as the general elicitors (Jones and Dangl 2006; Zipfel and Robatzek 2010). MAMPs/PAMPs are recognised by the extracellular transmembrane receptors (pattern recognition receptors—PRR) and trigger a non-specific, local immune response, termed PAMP-triggered immunity (PTI). The latter includes (1) the activation of the mitogen-activated protein kinase (MAP kinases) cascade and the transcriptional rearrangement of defence-related genes (e.g. ABCG transporters and pathogenesis-related (PR) proteins), (2) the generation of reactive oxygen species (ROS), (3) cell wall reinforcement and (4) the production and accumulation of antimicrobial compounds (phytoalexins) (Sasabe et al. 2002; Zipfel 2008). Pathogens capable of overcoming the constitutive barrier and suppressing PTI are defined as adapted (appropriate) pathogens (Aghnoum and Niks 2010; Ayliffe et al. 2011).

The second branch of the plant immune system constitutes effector-triggered immunity (ETI), which is activated by the recognition of pathogen effectors (avirulence factors) by plant resistance (R) proteins (Jones and Dangl 2006). It is believed that ETI is a faster and stronger version of PTI and is a major component of host resistance that effectively protects against race-specific pathogens. This immunity is in contrast to the so-called non-host resistance that protects the plant against a broad spectrum of pathogens. The non-host resistance is usually determined by many genes and involves constitutive and induced basal defence responses (Lipka et al. 2008; Jaulneau et al. 2010).

The phytohormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) act as defence signalling molecules. Depending on the lifestyle of the attacker, different hormones can be used to adapt the plant response to particular pathogens. Biotrophic pathogens are commonly sensitive to defence responses that are regulated by SA, whereas necrotrophs are regulated by JA and ET (Pieterse et al. 2009). Initially, the local defence response over time may extend to the entire plant and leads to the emergence of systemic acquired resistance (SAR). The establishment of SAR is associated with increased levels of SA. Another example of systemic resistance is the induced systemic resistance (ISR) triggered by the so-called plant growth-promoting rhizobacteria (PGPR), arbuscular mycorrhizal fungi and certain synthetic compounds. ISR requires the hormonal signals JA and ET and a faster or stronger activation of basal defence mechanisms when an induced plant is exposed to pathogens (Pieterse et al. 2009).

4 ABCGs Substrates and Defence

Historically, the major hallmark feature recognised as a consequence of the research on the full-size ABCG subfamily in particular was the involvement of at least certain members of the subfamily in plant–microbe interactions. It is believed that certain full-size ABCG transporters participate in non-specific defence reactions and confer non-host resistance. It has been shown that the expression of genes encoding ABCG transporters is induced by non-adapted pathogens, stress hormones (SA, JA, ET) and PAMPs. The dysfunction of these transporters increases the susceptibility of plants towards non-adapted pathogen infections. The potential defensive role of ABCGs is thought to be associated with the secretion of antifungal/antibacterial secondary metabolites and/or signalling molecules (Stein et al. 2006; Campbell et al. 2003; Sasabe et al. 2002).

A variety of low-molecular-weight compounds, called secondary metabolites, are engaged in the plant response to biotic and abiotic stresses, play important roles in the life cycle of the plants and mediate their interaction with the environment. Among these compounds, terpenoids were the first to be defined as substrates for plant ABCG transporters. The functional characterisation of NpPDR1/ABC1 of *Nicotiana plumbaginifolia*, a plasma membrane full-size ABCG transporter, revealed that its expression is highly induced by the tobacco-originated anti-fungal diterpene sclareol, which represents a chemical and non-specific line of defence in this plant. Transport assays with radiolabeled sclareolide, which is a structural analogue of sclareol, strengthened the notion that terpenoids might be substrates for full-size ABCGs (Jasinski et al. 2001). Recently, experiments in *Nicotiana tabacum* BY2 cells (cultivar Bright Yellow 2) overexpressing the plasma membrane full-size NtPDR1 revealed that this ABCG confers tolerance of BY2 towards several diterpenes, including sclareol, manool, abietic acid and dehydroabietic acid. The *NtPDR1*-overexpressing BY2 lines were also more effective in the extrusion of sclareol, manool and cembrene, as indicated by gas chromatography–mass spectrometry (GC–MS) method (Crouzet et al. 2013). Interestingly, several other ABCGs seem to be involved in sclareol transport, notably, *Arabidopsis* AtPDR12 (Campbell et al. 2003) and SpTUR2 from *Spirodela polyrhiza* (van den Brule et al. 2002); however, these plants do not produce endogenous sclareol.

In addition to defining the potential substrate spectra, studies on tobacco full-size ABCGs have driven the idea that these transporters are key components of plant defence. Indeed, preventing the expression of *NpPDR1* by RNAi interference caused an increased sensitivity of tobacco to infection with several fungal pathogens, including *Botrytis cinerea, Fusarium oxysporum f. sp. nicotianae, F. oxysporum f. sp. melonis* and *Rhizoctonia solani,* as well as to the oomycete pathogen *Phytophthora nicotianae* (Stukkens et al. 2005; Bultreys et al. 2009). Similarly, the silencing of *NtPDR1* expression caused increased susceptibility to the bacterial pathogen *Ralstonia solanacearum* (Seo et al. 2012). Recently, PDR5/ABCG5, which is a plasma membrane transporter from *N. tabacum,* has been shown to be involved in resistance to the specialist herbivore *Manduca sexta*

(Bienert et al. 2012). The proposed mode of action for participating in the secondary metabolite-based defence for tobacco ABCG transporters is the secretion/deposition of pathogen-deterring compounds on the leaf surface (Jasinski et al. 2001; Stukkens et al. 2005; Bienert et al. 2012).

The expression of several plant genes belonging to the full-size ABCG subfamily is induced by pathogens, e.g. *AtPDR12* (Campbell et al. 2003) and *MtABCG10* (Jasinski et al. 2009); pathogen-derived elicitors/PAMPS, e.g. *NtPDR1* (Sasabe et al. 2002), *MtABCG10* (Banasiak et al. 2013) and *AtPDR8* (Xin et al. 2013); insects, e.g. *NtPDR5*, (Bienert et al. 2012); or plant hormones that are implicated in the biotic stress response, such as SA and/or methyl jasmonate (MeJA). Notably, the expression of *OsPDR9, NpPDR1, NtPDR5* and *PgPDR3* is induced by MeJA (Grec et al. 2003; Moons 2008; Bienert et al. 2012; Zhang et al. 2013); *AtPDR8* and *OsPDR20* are induced by SA (Kobae et al. 2006; Stein et al. 2006; Moons 2008); and *GmPDR12* and *AtPDR12* are induced by both agents (Campbell et al. 2003; Eichhorn et al. 2006). Moreover, recent transcription profiling of *Arabidopsis* full-size ABCGs revealed that half of them positively responded to JA and/or SA. When half-size ABCGs were investigated, half of them were induced by JA but only one-fourth were induced by SA (Kang et al. 2011).

In *Arabidopsis*, the importance of ABCGs for defence is exemplified by the fact that the loss of a full-size plasma membrane transporter, known as AtPDR8 (synonyms PEN3, ABCG36), causes hypersensitive cell death upon infection with the virulent bacterial pathogen *Pseudomonas syringae* (Kobae et al. 2006) and results in susceptibility towards inappropriate pathogens (*Blumeria graminis* f. sp. *hordei*, *Erysiphe pisi, Phytophthora infestans*) (Stein et al. 2006). Interestingly, the infection process by pathogenic fungi and the treatment of *Arabidopsis* with a bacterial elicitor such as the flagellin-derived peptide flg22 induces the allocation of AtPDR8 towards the site of infection (Stein et al. 2006; Xin et al. 2013). This observation supports the idea that AtPDR8 exports a defence compound(s) in a focal manner at attempted invasion sites. AtPDR8 accumulation/recruitment requires its corresponding PRR receptors, namely, FLS2 (flagellin-sensing 2) and CERK1 (chitin elicitor receptor-like kinase1), but does not require the BAK1 co-receptor (BRI1-associated receptor kinase 1) (Underwood and Somerville 2013). The focal accumulation of AtPDR8 shows differential sensitivity to specific pharmacological inhibitors that affect the actin cytoskeleton, microtubules or secretory trafficking, indicating distinct mechanisms for AtPDR8 recruitment to the host–pathogen interface. Focal accumulation requires actin but is not affected by inhibitors of microtubule polymerisation or secretory trafficking (Underwood and Somerville 2013).

A constitutive expression level that was not responsive to pathogen inoculation was observed for another representative of full-size ABCG transporters that is important for plant defence, known as Lr34 from *Triticum aestivum* (bread wheat). The *Lr34* (Leaf Rust 34) gene was identified as a functional allele that confers durable resistance against *Puccinia triticina*, *Puccinia striiformis* and *B. graminis*. Because of its durability and ability to restrict the growth of multiple pathogens, *Lr34* has become one of the most important, non-specific resistance

gene to multiple pathogens in wheat breeding, and it has been incorporated into more than 50 % of wheat cultivars around the world (Krattinger et al. 2009).

ABCG transporters that are possibly involved in defence responses have been identified in the representatives of legumes, which are the second-most important crop family after grasses. To date, the systematic identification of the legume ABCG members has been conducted in *L. japonicus* and *M. truncatula* (Sugiyama et al. 2006; Jasinski et al. 2009). Most of the identified *Medicago* full-size G genes are expressed in roots, and the number expressed in other organs is markedly smaller (Jasinski et al. 2009). A similar observation was made by Sugiyama and coworkers regarding the *L. japonicus ABCG* genes (Sugiyama et al. 2006). The rhizosphere is a place of intense interactions with bacteria, fungi and insects as well as other plants, and efficient transport through cellular membranes is necessary to ensure the effective flow of different molecules (e.g. root exudates with defence or signalling properties). Indeed, the dysfunction of root-expressed, full-size ABCG transporters (AtABCG30/PDR2, AtABCG34/PDR6 and AtABCG35/AtPDR7) results in a change in soil microflora, possibly as a consequence of altered root exudate composition in the mutants (Badri et al. 2008, 2009).

Infection of *M. truncatula* roots with *Fusarium culmorum,* which is a necrotrophic, soil-borne, pathogenic fungus, results in an increased transcript accumulation of two full-size *ABCGs*, namely, *MtABCG10* and *MtABCG16*. Both transcripts also strongly accumulated in leaves infected with the host-specific fungus *Phoma medicaginis,* suggesting a relation between their activation and fungal infections. Interestingly, the expression of the *MtABCG10* gene is also upregulated in roots upon infection by the symbiotic bacteria *Sinorhizobium meliloti,* and it can be hypothesised that certain legume ABCG proteins are involved in plant–fungus interactions and in plant–symbiont associations. Accordingly, several *L. japonicus* full-size ABCGs, which are homologues of *MtABCG10,* were induced in roots infected with their symbiotic partner *Mesorhizobium loti* (Sugiyama et al. 2006).

Further studies on the ABCG10 from *M. truncatula* revealed that its transcript was present in the vascular tissue of different organs, and the corresponding protein has been found in the plasma membrane. The expression of this transporter was also upregulated upon the treatment of roots with PAMPs (fungal cell wall oligosaccharides). The accumulation of *MtABCG10* mRNA followed that of transcripts of genes that coded enzymes from the phenylpropanoid pathway, namely, phenylalanine ammonia-lyase (PAL) and isoflavone synthase (IFS), and the silencing of *MtABCG10* in hairy roots resulted in a lower accumulation of a phenylpropanoid pathway-derived phytoalexin medicarpin as well as its precursors, such as liquiritigenin and isoliquiritigenin (Banasiak et al. 2013). The phenylpropanoid pathway products play a particular role in biotic stress responses. This multifaceted group of plant secondary products can function as antimicrobial agents, UV protectants, pollinator attractants, floral pigments and inducers of the nodulation genes in symbiotic soil bacteria. Among these compounds, isoflavonoids are limited primarily to the *Leguminosae*. Isoflavonoids are thought to represent the majority of the phytoalexins that are produced by legume plants (Hassan and Mathesius 2012).

For instance, soybean partial resistance to *Fusarium solani* seems to be associated with the ability of soybean roots to produce phytoalexin glyceollin in response to fungal infection (Lozovaya et al. 2004).

As a consequence of reduced amounts of medicarpin, infections of *Medicago* roots with *F. oxysporum,* which is a root-infecting, non-specific pathogen, progress faster in *MtABCG10*-silenced lines than in wild-type plants. The expression of *MtABCG10* is not influenced by signalling molecules such as SA or MeJA, a result that differs from the effect observed for other legume full-size members of the ABCG subfamily, namely, *PDR12*, which was identified in soybean (*G. max*). The expression of this gene has been rapidly induced by SA, but, to date, little is known about the possible function(s) of *GmPDR12 in planta* (Eichhorn et al. 2006).

It is thought that in *Medicago* PAMPs cause the *de novo* synthesis of medicarpin and that MeJA stimulates the release of its conjugate (glycosylated, malonylated) precursors from the vacuole (Naoumkina et al. 2007; Farag et al. 2008). MtABCG10 is a plasma membrane transporter, and the expression of its corresponding gene is highly induced by PAMPs but not MeJA. Silencing the expression of this gene results in decreased amounts of free medicarpin precursors (aglycones) but does not influence the levels of their conjugates. Thus, MtABCG10 may be a modulator of *Medicago* phytoalexin levels during the defence response that is associated with de novo synthesis (Banasiak et al. 2013). The research on legume ABCGs, together with that on *Arabidopsis* ABCG29, which is a p-coumaryl alcohol transporter (Alejandro et al. 2012), suggest that members of the G subfamily can have a complex impact on phenolic compounds in plants.

5 Plants and Symbioses

Many plant species establish interactions with microorganisms that are advantageous to both partners. The best studied symbiotic associations include the legume–rhizobia symbiosis (LRS) between legumes and nitrogen-fixing bacteria, which are collectively called rhizobia, as well as arbuscular mycorrhizae (AM) between most terrestrial plants and fungi of the phylum *Glomeromycota* (Oldroyd 2013). Whereas AM fungi can colonise the majority of land plants (a few plant families have species that do not form mycorrhizal associations, e.g. *Brassicaceae, Chenopodiaceae*), root nodule symbiosis with rhizobia almost exclusively involves legumes (*Fabaceae*). AM originated approximately 450 Ma ago (Kistner and Parniske 2002) and is thought to be essential for plant survival in harsh environments. As a consequence of root colonisation by AM fungi, the plant gains access to nutrients such as phosphorus (P), nitrogen (N), sulphur (S), zinc (Zn) and copper (Cu), whereas, in LRS, the plant is provided with only nitrogen (N) (Prell and Poole 2006).

The initial phase of symbioses represents a chemical dialogue between partners prior to direct contact and root colonisation. Environmental factors such as N or P shortages promote secretion of signalling molecules by plant roots, which leads to the attraction of rhizobia or AM fungi. Plant secondary metabolites, including flavonoids and the plant hormones strigolactones, play such an attractant role (Oldroyd 2013). In turn, symbiotic partners release signalling molecules, such as the rhizobial Nod factors and mycorrhizal Myc factor. These diffusible signals represent lipochito-oligosaccharides (LCOs) are recognised by the host plants and trigger a common symbiosis (SYM) pathway (Oldroyd 2013). The latter pathway is composed of genes that encode, *inter alia,* a receptor kinase that is localised to the plasma membrane, components of signal transduction to the nucleus and a nuclear CCaMK (calcium and calmodulin-dependent protein kinase) (Parniske 2008; Oldroyd et al. 2011; Singh and Parniske 2012). The activation of this pathway results in the formation of nodules or promotes root invasion.

Flavonoids, which are released by plants to the rhizosphere during the pre-symbiotic stage, function as chemo-attractants that concentrate the compatible rhizobia at the root surface (Dharmatilake and Bauer 1992). Rhizobia enter the plant root by infection threads that are formed as invaginations of the plant cell, enabling the invasion of the rhizobia into the root tissue. Finally, nodules initiate below the site of bacterial infection, and, in this specialised organ, rhizobia convert atmospheric nitrogen effectively into ammonium.

Strigolactones are released from plant roots and stimulate hyphal branching in AM fungi (Akiyama et al. 2005). These molecules stimulate AM fungal metabolism (Besserer et al. 2006) and, at the same time, inhibit the growth of fungal pathogens (Dor et al. 2011). The AM fungus colonises the plant root cortex through intercellular hyphal growth, and tree-shaped structures called arbuscules are formed in the inner root cortical cells.

6 ABCG Substrates and Symbioses

The initial steps of both beneficial types of interactions, LRS and AM, are influenced by ABC transporters. Currently, the transporters involved in flavonoid secretion into the rhizosphere are unknown, but biochemical analyses suggest that plasma membrane ABC-type proteins can be implicated in the release of such molecules. Genistein and daidzein are the major flavonoids in soybean root exudates that act as legume-to-*Rhizobium* signal molecules (Kosslak et al. 1987; Smit et al. 1992). Sugiyama and coworkers, using plasma membrane vesicles from soybean, provided evidence that the export of genistein is ATP dependent. Based on the type-specific inhibitors of membrane transporters that the authors used in this work, it was suggested that a member of the G subfamily might be a candidate as a genistein exporter. However, the recognition of the molecular nature of this transporter remains undetermined (Sugiyama et al. 2007, 2008).

Recently, it was proposed that an ABC protein belonging to the G subfamily, the PDR1 from *Petunia hybrida,* participated in strigolactones exudation to the rhizosphere (Kretzschmar et al. 2012). *PhPDR1* is expressed preferentially during P starvation, a condition that favours AM. PhPDR1 is localised to the plasma membrane of sub-epidermal passage cells, which are the preferred entry point for AM fungi. Additionally, the mRNA of *PhPDR1* increases after inoculation with the mycorrhizal fungi *Glomus intraradices* as well as after treatment with the synthetic strigolactone GR24. Moreover, the *phpdr1* mutants are defective in strigolactones exudation from their roots, leading to reduced symbiotic association (Kretzschmar et al. 2012).

The ABCG transporters also act at the later stages of AM symbiosis, i.e. during arbuscule development. Arbuscules are surrounded by a peri-arbuscular membrane (PAM), which is a continuation of the plant cell plasma membrane but with unique protein composition and biological properties. One of the fundamental differences that distinguish PAM is the presence of highly specific membrane transporters that mediate the transport of nutrients and signalling molecules between AM partners (Gaude et al. 2012). Half-size ABCG transporters (STR1 and STR2) were found to be indispensable for arbuscule development in *M. truncatula* and *O. sativa* (Zhang et al. 2010; Gutjahr et al. 2012). STRs are representatives of the ABCG subfamily and are found in vascular plants but are absent in *Arabidopsis* and other species that do not form mycorrhizal associations. Both *STR* genes are expressed constitutively in vascular tissue. Upon mycorrhization, the level of *STR* mRNAs increases in cortical cells containing arbuscules.

The dysfunction of STRs in *M. truncatula* and in *O. sativa* results in a stunted arbuscule phenotype; however, hyphopodium formation and fungal entrance to the cortex occur normally. Interestingly, the *M. truncatula str* mutant is not deficient in nodulation, suggesting that STRs function downstream of the common symbiosis pathway. STR1 and STR2 form a heterodimer that is localised to the peri-arbuscular membrane. It is thought that the STR1/STR2 complex releases non-defined molecule(s) from the cortical cell to the peri-arbuscular apoplastic space and thereby influences arbuscule growth. Researchers have suggested that STR1/STR2 might transport signal molecules, locally required nutrients or precursors of plant-derived structural components that are essential for AM fungus (Zhang et al. 2010; Gutjahr et al. 2012).

Large-scale transcriptomics of various legume species allow for the identification of the genes encoding ABC transporters, the expression of which changes temporally and spatially during AM- and/or LR-symbiotic associations. For instance, a global gene expression analysis of *M. truncatula* roots inoculated with the AM fungi *G. intraradices* and *Glomus mosseae* showed that the expression of six genes encoding ABC transporters were induced by more than tenfold when compared to non-inoculated roots (Hogekamp et al. 2011). Among these genes was a half-size *ABCG* gene (Medicago GeneChip ID: Mtr.52071.1.S1_at/MtWBC5) that was exclusively expressed in arbuscule-containing cells. The second-highest upregulated *ABCG* gene upon *Medicago* root colonisation by *Glomus. spp.* corresponded to the previously described AM-related STR2 (Zhang et al. 2010;

Hogekamp et al. 2011). Similarly, when *L. japonicus* was inoculated with *Glomus margarita,* homologues of the *STR* genes and *MtWBC5* were found to be strongly upregulated during AM symbiosis (Gutjahr et al. 2009).

In *Medicago*, it was shown that the expression of genes encoding full-size ABCG transporters, possibly associated with defence reactions; for example, *MtABCG10* was significantly suppressed during later stages of mycorrhizal colonisation. The presence of MtABCG10 in *Medicago* roots has been shown to be crucial for isoflavonoid-derived phytoalexin biosynthesis and, consequently, the efficiency of defence responses (Hogekamp et al. 2011; Banasiak et al. 2013). Defence responses must be controlled upon root colonization and/or symbiotic partners co-existence. Therefore, the suppression of MtABCG10 is consistent with the observation that transcripts of many defence- and stress-response genes are down-regulated when the AM is completely established (Liu et al. 2003). Moreover, transcriptomic data concerning the early phase of AM demonstrate that the expression of eight *ABCG* (e.g. *MtWBC15* and *MtPDR14*) genes increased by at least twofold in *M. truncatula* root epidermal cells that were contacted by AM-fungal hyphopodia (Ortu et al. 2012).

Correspondingly, a number of transcriptomic studies have been conducted to identify genes encoding transporters potentially involved in LR symbiosis. A combination of Affymetrix Medicago GeneChips and laser-capture microdissection was used to determine the *Medicago* gene expression profiles of specific cells/tissues that were derived from different parts of the nodules (Limpens et al. 2013). *Medicago* possesses indeterminate nodules (with a persistent meristem) in which several zones can be distinguished based on morphology and function: zone I consists of apical meristem; zone II is the infection zone, where rhizobia are released from the infection threads to the plant cells and are subsequently surrounded by the plant-derived symbiosome membrane; zone III is the fixation zone, where plant cells containing nitrogen-fixing bacteria are located; and zone IV is where the senescence of bacteria and host cells occurs (Oldroyd and Downie 2008).

Among the *Medicago* genes, especially those expressed in zone I, *MtPDR18* exhibits a greater than 13-fold change when compared to un-infected control tissue (Limpens et al. 2013). Eleven *ABC* genes showed altered expression in zone II, of which three genes (e.g. *MtPDR10, MtPDR16, MtPDR18*) coded for the closest homologues of the *Arabidopsis* defence-related, full-size transporters AtPDR12 and AtPDR8. Interestingly, the expression of these three *Medicago* homologues in zone II was down regulated (Damiani et al. 2012). This observation supports the hypothesis that the suppression of defence responses in infected cells is crucial for rhizobia accommodation, similar to what was shown for AM symbiosis (Liu et al. 2003; Kouchi et al. 2004). Moreover, nine genes coding ABC transporters, two belonging to the ABCG subfamily, have been reported as particularly expressed in the un-infected cells that were attached to symbiosomes that contain cells in the fixation zone of nodules (Limpens et al. 2013). The proposed functions of these cells are to support the metabolism of infected cells, to mediate the nutrient exchange between the plant and the bacteroids and to protect infected cells from

oxygen by producing the suberin barrier (Hartmann et al. 2002). High numbers of upregulated *ABC* genes were found in cells containing symbiosomes; however, none of them coded for ABCG transporters (Limpens et al. 2013).

Interestingly, among the *ABC* genes whose expression levels increased at least twice under the influence of the Nod factor (early stage of LRS), three belong to the ABCG subfamily (Czaja et al. 2012). All of these *ABCGs* are expressed exclusively in roots and do not have close homologues in non-legume plant species. Based on transcriptomic data, it can be assumed that ABCG transporters play a particular role at the early stages of symbioses (partner recognition) and are then down-regulated during colonisation (plant defensive response attenuation) finally these transporters are responsible for efficient nutrient/signal exchange upon symbiotic partner co-existence. Cumulatively, these characteristics illustrate the possible functional diversity of the ABCG transporter subfamily at different stages of symbiotic associations.

7 ABCG Distribution in Legumes

Legumes (*Fabaceae*) are the third-largest group of flowering plants (Young and Bharti 2012). Because of their symbiosis with nitrogen-fixing bacteria and mycorrhizal associations, legumes are not dependent upon nitrogen fertilisers and can efficiently acquire nutrients, especially phosphate, from the soil. The co-cultivation of leguminous plants with other crops can increase the yield of the latter under limited fertiliser input (Davis et al. 2012). Legumes are also an inexpensive source of high-quality proteins for humans and animals as well as of natural substances with medicinal properties, such as isoflavones (Dixon and Sumner 2003). The economic importance of *Fabaceae* and their role in understanding symbiotic associations, defence responses and the interplay between these two types of interactions underlines the necessity of a model legume species for basic research.

M. truncatula, L. japonicus and *G. max* have been the subjects of extensive genomic DNA sequencing over the past few years, making them suitable models for (1) genome structure and transcriptional landscape studies, (2) finding syntenic relationships among *Fabaceae* members and (3) gene discovery (Cannon 2013). This knowledge has a profound impact on agriculture because it allows for associations between casual DNA sequences and particular phenotypes that are important for crop production and/or symbiosis efficiency. One of the key issues in symbioses is the chemical crosstalk between partners, which concerns pre-symbiotic interactions as well as the later co-existence of symbionts. For instance, colonised plant cells are highly compartmentalised and have a complex membrane system that is specialised for the diverse functions in molecular communication and nutrient exchange mediated by a set of transporters.

Genome sequencing projects of model legume plants have allowed for the systematic identification and classification of genes encoding potential membrane transporters and might facilitate the functional characterisation of these transporters

in the near future (Sugiyama et al. 2006; Jasinski et al. 2009; Benedito et al. 2010). Among the identified transporters are representatives of the ABCG protein family (Figs. 1 and 2); in addition, in light of recently conducted experiments, including microarrays, at least certain members of the G subfamily of ABC transporters appear to be symbiosis related (Sugiyama et al. 2007, 2008; Zhang et al. 2010; Limpens et al. 2013). Nonetheless, only a few transporters have been characterised at the functional level (Zhang et al. 2010; Banasiak et al. 2013). Compared with *A. thaliana,* which has 15 full-size ABCGs (PDRs), *M. truncatula* and *G. max* have significantly more PDRs, with 24 and 29, respectively. Interestingly, the number of WBC proteins, except for those in *G. max,* is similar among *A. thaliana*, *M. truncatula* and *L. japonicus* (28, 25 and 24, respectively) (Sugiyama et al. 2006; Verrier et al. 2008). The high number of ABCG transporter-coding genes, especially those coding WBCs (52 members) in *G. max,* resulted from whole-genome duplication events. Seventy-four per cent of *G. max* WBC genes possess close paralogues that form gene pairs (Fig. 3), and this finding is consistent with the observation that nearly 75 % of genes in this species are present in multiple copies (Schmutz et al. 2010; Cannon 2013).

Phylogenetic analyses of ABCG transporters (both WBC and PDR, see Figs. 3 and 4) from *M. truncatula, G. max* and *L. japonicus* and those that do not form symbiotic associations, such as *Arabidopsis*, revealed species-specific protein expansions. This characteristic is exemplified by the following subgroups: (1) *A. thaliana* (AtWBC1, 2, 6, and 16–20; AtPDR2 and 13–15), (2) *M. truncatula* (MtWBC4-9; MtPDR1-4 and 20, 21) and (3) *G. max* (GmWBC11-17; GmPDR1-3). Such high protein redundancy/clustering may indicate that the duplication of certain ABCG genes has occurred as a result of adaptation to new functions and/or the transport of new substrates.

Furthermore, a phylogenetic tree of the PDRs (Fig. 4) shows the presence of a unique clade consisting exclusively of legume sequences. Interestingly, homologues of transporters from this clade are also present in other legumes, e.g. C*icer arietinum*, but not in *Arabidopsis* or other non-legume plants for which genomic sequences are available (e.g. *Vitis vinifera, Cucumis sativus, Solanum lycopersicum*, *O. sativa, Sorghum bicolor* and *Selaginella moellendorffii*). This observation supports the assumption of the "legume" nature of this clade and the possible divergence that occurred after the appearance of *Fabaceae*. The clustering of PDRs that are potentially associated with legume-specific functions is especially apparent for *M. truncatula*. All nine *Medicago* genes (MtPDR1-6 and 20–22) encoding PDRs that belong to the legume clade (Fig. 4) are located within 300 kb of each other on chromosome 7. These PDRs show high amino acid identity among their corresponding protein sequences (between 62 and 93 %), and the corresponding genes have similar exon/intron patterns, indicating that they most likely arose through tandem duplication, which is a well-known mechanism leading to the evolution of multigene families in *Medicago* (Young et al. 2011; Cannon 2013). Among them are genes that are exclusively expressed in roots (e.g. *MtPDR3*, *MtPDR4*) (Jasinski et al. 2009) and whose expression is upregulated by diffusible signals from rhizobial bacteria (NOD factors) (e.g. *MtPDR4*, *MtPDR20*) (Czaja

*Medicago truncatula**		*Glycine max*				*Lotus japonicus*	
Gene name	**Accession**	**Gene name**	**Accession**	**Gene name**	**Accession**	**Gene name**	**Accession**
MtWBC1/MtSTR1	XP_003631132	GmWBC1a/GmSTR1a	XP_003530969	GmWBC15	XP_003531028	LjWBC1/LjSTR1	chr4.CM0042.2570.r2.d
MtWBC2/MtSTR2	XP_003612949	GmWBC1b/GmSTR1b	XP_003525183	GmWBC16	XP_003532608	LjWBC2/LjSTR2	chr2.CM0177.350.r2.m
MtWBC3	XP_003617868	GmWBC2a/GmSTR2a	Glyma01g02440.1**	GmWBC17a	XP_003532610	LjWBC3	chr5.CM0180.730.r2.m
MtWBC4	XP_003606172	GmWBC2b/GmSTR2b	XP_003533431	GmWBC17b	XP_003541427	LjWBC4	chr1.CM0012.320.r2.m
MtWBC5	XP_003608439a	GmWBC3a	XP_003535589	GmWBC18a	Glyma20g26160.1**	LjWBC5	chr3.CM0111.520.r2.a
MtWBC6	XP_003608439b	GmWBC3b	XP_003556131	GmWBC18b	XP_003536632	LjWBC6	chr6.LjT111F18.90.r2.m
MtWBC7	XP_003608436	GmWBC4a	XP_003539614	GmWBC19a	XP_003537002	LjWBC7	chr1.CM0029.820.r2.m
MtWBC8	XP_003608435	GmWBC4b	XP_003543218	GmWBC19b	XP_003541425	LjWBC8	chr3.CM0590.360.r2.d
MtWBC9	XP_003608431	GmWBC5a	Glyma08g00280.1**	GmWBC20a	XP_003539196	LjWBC9	chr2.CM0803.900.r2.a
MtWBC10	XP_003630284	GmWBC5b	XP_003525127	GmWBC20b	XP_003540763		
MtWBC11	XP_003607356	GmWBC6a	XP_003522483	GmWBC21	XP_003518985		
MtWBC12	XP_003612480	GmWBC6b	XP_003528053	GmWBC22a	XP_003535522		
MtWBC13	XP_003625526	GmWBC7a	XP_003540196	GmWBC22b	XP_003555441		
MtWBC14	XP_003609516	GmWBC7b	XP_003543454	GmWBC23a	XP_003519640		
MtWBC15	XP_003592180	GmWBC8a	XP_003537734	GmWBC23b	XP_003544606		
MtWBC16	XP_003602630	GmWBC8b	XP_003540269	GmWBC24	XP_003552990		
MtWBC17	XP_003607359	GmWBC9a	XP_003537732	GmWBC25a	XP_003521505		
MtWBC18	XP_003607361	GmWBC9b	XP_003540615	GmWBC25b	XP_003553629		
MtWBC19	XP_003638054	GmWBC10a	XP_003534067	GmWBC26a	XP_003519092		
MtWBC20	XP_003591854	GmWBC10b	XP_003548275	GmWBC26b	XP_003535833		
MtWBC21	XP_003591865	GmWBC11a	XP_003532606	GmWBC27a	XP_003535544		
MtWBC22	XP_003590459	GmWBC11b	XP_003543927	GmWBC27b	XP_003555426		
MtWBC23	XP_003625668	GmWBC12	XP_003531027	GmWBC28a	XP_003535512		
MtWBC24	XP_003592545	GmWBC13	XP_003543921	GmWBC28b	XP_003556255		
MtWBC25	XP_003592758	GmWBC14a	XP_003532607	GmWBC29a	XP_003517105		
		GmWBC14b	XP_003543926	GmWBC29b	XP_003537708		

Other species	
Gene name	**Accession**
CaSTR1	XP_004503258
CsSTR1	XP_004163819
VvSTR1	XP_002278856
OsSTR1	NP_001063119
SbSTR1	XP_002445326
CaSTR2	XP_004512587
SbSTR2	XP_002458373
VvSTR2	CAN62797
OsSTR2	BAD30878

Fig. 1 Accession numbers of plant half-size ABCG proteins (WBCs). *Medicago truncatula* (Mt), *Glycine max* (Gm), *Cicer arietinum* (Ca), *Cucumis sativus* (Cs), *Vitis vinifera* (Vv), *Oryza sativa* (Os), *Sorghum bicolour* (Sb) accession numbers are from GenBank (http://www.ncbi.nlm.nih.gov/genbank/). *Lotus japonicus* (Lj) accession numbers are from database miyakogusa.jp 2.5 (http://www.kazusa.or.jp/lotus/). *MtWBC1-16 corresponds to MtABCG1-16 in Zhang et al. (2010). **G. *max* (Gm) accession numbers are from *Glycine max* Genome DB (http://www.plantgdb.org/GmGDB/)

*Medicago truncatula**		*Glycine max*				*Lotus japonicus*	
Gene name	**Accession**	**Gene name**	**Accession**	**Gene name**	**Accession**	**Gene name**	**Accession**
MtPDR1	XP_003625399	GmPDR1a/GmPDR12**	NP_001237697	GmPDR18	XP_003544291	LjPDR1	chr1.CM0295.1190.r2.a
MtPDR2	XP_003625398	GmPDR1b	XP_003553512	GmPDR19	XP_003551279	LjPDR2	chr1.CM0295.1210.r2.a
MtPDR3	XP_003625401	GmPDR2	XP_003553513	GmPDR20a	XP_003550575	LjPDR3	chr5.CM0345.1620.r2.m
MtPDR4	XP_003625361	GmPDR3	XP_003553514	GmPDR20b	XP_003528598	LjPDR4	chr5.CM0345.1640.r2.m
MtPDR5	XP_003625405	GmPDR4a	XP_003550166	GmPDR21	XP_003550950	LjPDR5	chr3.CM0226.110.r2.m
MtPDR6	XP_003625403	GmPDR4b	XP_003545503			LjPDR6	chr3.CM0226.120.r2.m
MtPDR7	XP_003588698	GmPDR5a	XP_003526427			LjPDR7	chr2.CM0346.80.r2.m
MtPDR8	XP_003588699	GmPDR5b	XP_003522631			LjPDR8	chr3.CM0213.540.r2.m
MtPDR9	XP_003597816	GmPDR6	XP_003546230				
MtPDR10	XP_003597819	GmPDR7a	XP_003546218				
MtPDR11	XP_003597818	GmPDR7b	XP_003543663				
MtPDR12	XP_003625772	GmPDR8	XP_003528365				
MtPDR13	XP_003625773	GmPDR9	XP_003529755				
MtPDR14	XP_003625775	GmPDR10	XP_003546211				
MtPDR15	XP_003609865	GmPDR11a	XP_003549791				
MtPDR16	XP_003627034	GmPDR11b	XP_003524521				
MtPDR17	XP_003615634	GmPDR12	XP_003520687				
MtPDR18	XP_003597683	GmPDR13	XP_003554421				
MtPDR19	XP_003609200	GmPDR14	XP_003520263				
MtPDR20	XP_003625363+XP_003625364	GmPDR15a	XP_003555459				
MtPDR21	XP_003625365+XP_003625367	GmPDR15b	XP_003535502				
MtPDR22	XP_003625414	GmPDR16a	XP_003530098				
MtPDR23	XP_003603455	GmPDR16b	XP_003531649				
MtPDR24	XP_003625777	GmPDR17	XP_003543626				

Other species	
Gene name	**Accession**
NpPDR1	Q949G3.1
NpPDR2	Q2PCF1.1
NtPDR1	Q76CU2.1
NtPDR3	Q5W274.1
SpTUR2	O24367.1
PhPDR1	AFA43816.1

Fig. 2 Accession numbers of plant full-size ABCG proteins (PDRs). *Medicago truncatula* (Mt), *Glycine max* (Gm), *Nicotiana plumbaginifolia* (Np), *Nicotiana tabacum* (Nt), *Spirodela polyrrhiza* (Sp), *Petunia hybrida* (Ph) accession numbers are from GenBank (http://www.ncbi.nlm.nih.gov/genbank/). *Lotus japonicus* (Lj) accession numbers are from database miyakogusa.jp 2.5 (http://www.kazusa.or.jp/lotus/). *MtPDR1-19 corresponds to MtABCG1-19 in Jasinski et al. (2009). **In Eichhorn et al. (2006) GmPDR1a is called GmPDR12

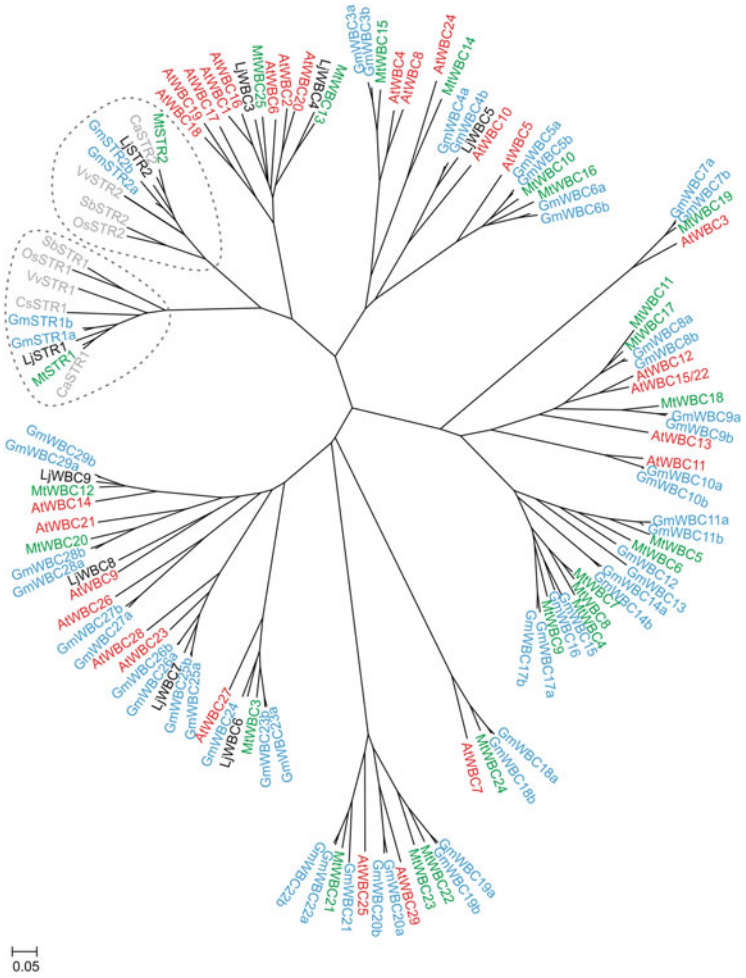

Fig. 3 Unrooted phylogenetic tree of the half-size ABCG proteins (WBCs) of *Arabidopsis thaliana* (At), *Medicago truncatula* (Mt), *Glycine max* (Gm), *Lotus japonicus* (Lj), with a focus on STR1 and STR2 and their orthologs from *Cicer arietinum* (Ca), *Cucumis sativus* (Cs), *Vitis vinifera* (Vv), *Oryza sativa* (Os), *Sorghum bicolor* (Sb) (broken borders). *A. thaliana* accession numbers are from Verrier and associates (2008), other plants accession numbers are shown in Fig. 1. The full-length protein sequences were aligned using CLUSTAL_W and the phylogenetic tree was constructed using the neighbour-joining method

et al. 2012). In *Medicago,* a high level of DNA recombination has been associated with gene clusters belonging to the multigene families that are related to biotic interactions (Paape et al. 2012).

Within the WBC transporters, two independent and well-distinguished clades that are characterised by the presence of STR1 and STR2 homologues can be found (Fig. 3). Both clades can be considered AM-related because their members include

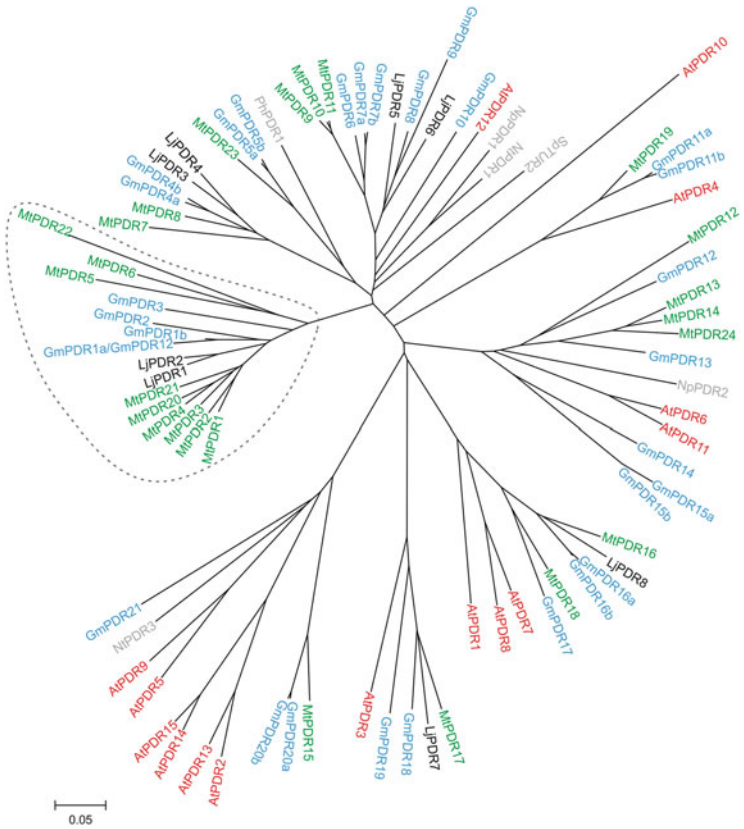

Fig. 4 Unrooted phylogenetic tree of the full-size ABCG proteins (PDRs) of *Arabidopsis thaliana* (At), *Medicago truncatula* (Mt), *Glycine max* (Gm), *Lotus japonicus* (Lj), *Nicotiana plumbaginifolia* (Np), *Nicotiana tabacum* (Nt), *Spirodela polyrrhiza* (Sp), *Petunia hybrida* (Ph), with focus on legume-specific clade described in the text (broken border). *A. thaliana* accession numbers are from Verrier and associates (2008), other plants accession numbers are shown in Fig. 2. The full-length protein sequences were aligned using CLUSTAL_W and the phylogenetic tree was constructed using the neighbour-joining method

STR1 and STR2 from *M. truncatula* and *O. sativa* and were recognised as crucial for mycorrhizal arbuscule development. Interestingly, these clades comprise only one-to-one orthologues (no species-specific expansion) from different vascular plants. These observations might indicate a functional conservation of these proteins between members of the di- and monocotyledons and might hint at the expected function of STRs in other plants (Zhang et al. 2010; Gutjahr et al. 2012).

8 A Final Word

Important details concerning plant ABC transporters have as yet only been investigated in a few plants due to the favour of an experimental object, the basic interest of researchers or by chance. We have only just begun to recognise these proteins in legumes. It might be obligate to postulate that the redundancy of ABCG genes in legumes is relevant for the recognition between symbiotic and pathogenic microorganisms and for establishing specific types of interactions. However, a precise role of these transporters in symbioses and the transport of, e.g. signalling molecules remain to be discovered. Studying membrane transporters contributes to the understanding of how fluxes of molecules and their biosyntheses are integrated into programs of plant cell/tissue specialisation that ensure their tight connection with biological events such as defence responses or symbioses. It is likely that understanding transport processes in plants will result in an increased resistance towards pathogens and in their more efficient nodulation and mycorrhization, which, in turn, will increase the production of high-quality plant material without harming the environment.

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References

Aghnoum R, Niks RE (2010) Specificity and levels of nonhost resistance to nonadapted *Blumeria graminis* forms in barley. New Phytol 185(1):275–284. doi:10.1111/j.1469-8137.2009.03039.x

Akiyama K, Matsuzaki K, Hayashi H (2005) Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. Nature 435(7043):824–827. doi:10.1038/nature03608

Alejandro S, Lee Y, Tohge T, Sudre D, Osorio S et al (2012) AtABCG29 is a monolignol transporter involved in lignin biosynthesis. Curr Biol 22(13):1207–1212. doi:10.1016/j.cub.2012.04.064

Anjard C, Loomis WF, Dictyostelium Sequencing C (2002) Evolutionary analyses of ABC transporters of *Dictyostelium discoideum*. Eukaryot Cell 1(4):643–652

Ayliffe M, Devilla R, Mago R, White R, Talbot M et al (2011) Nonhost resistance of rice to rust pathogens. Mol Plant-Microbe Interact 24(10):1143–1155. doi:10.1094/MPMI-04-11-0100

Badri DV, Loyola-Vargas VM, Broeckling CD, De-la-Pena C, Jasinski M et al (2008) Altered profile of secondary metabolites in the root exudates of Arabidopsis ATP-binding cassette transporter mutants. Plant Physiol 146(2):762–771. doi:10.1104/pp. 107.109587

Badri DV, Quintana N, El Kassis EG, Kim HK, Choi YH et al (2009) An ABC transporter mutation alters root exudation of phytochemicals that provoke an overhaul of natural soil microbiota. Plant Physiol 151(4):2006–2017. doi:10.1104/pp. 109.147462

Banasiak J, Biala W, Staszkow A, Swarcewicz B, Kepczynska E et al (2013) A *Medicago truncatula* ABC transporter belonging to subfamily G modulates the level of isoflavonoids. J Exp Bot 64(4):1005–1015. doi:10.1093/jxb/ers380

Benedito VA, Li H, Dai X, Wandrey M, He J et al (2010) Genomic inventory and transcriptional analysis of *Medicago truncatula* transporters. Plant Physiol 152(3):1716–1730. doi:10.1104/pp. 109.148684

Besserer A, Puech-Pages V, Kiefer P, Gomez-Roldan V, Jauneau A et al (2006) Strigolactones stimulate arbuscular mycorrhizal fungi by activating mitochondria. PLoS Biol 4(7):e226. doi:10.1371/journal.pbio.0040226

Bienert MD, Siegmund SE, Drozak A, Trombik T, Bultreys A et al (2012) A pleiotropic drug resistance transporter in Nicotiana tabacum is involved in defense against the herbivore Manduca sexta. Plant J 72(5):745–757. doi:10.1111/j.1365-313X.2012.05108.x

Bird D, Beisson F, Brigham A, Shin J, Greer S et al (2007) Characterization of Arabidopsis ABCG11/WBC11, an ATP binding cassette (ABC) transporter that is required for cuticular lipid secretion. Plant J 52(3):485–498. doi:10.1111/j.1365-313X.2007.03252.x

Bultreys A, Trombik T, Drozak A, Boutry M (2009) Nicotiana plumbaginifolia plants silenced for the ATP-binding cassette transporter gene NpPDR1 show increased susceptibility to a group of fungal and oomycete pathogens. Mol Plant Pathol 10(5):651–663. doi:10.1111/j.1364-3703.2009.00562.x

Campbell EJ, Schenk PM, Kazan K, Penninckx IA, Anderson JP et al (2003) Pathogen-responsive expression of a putative ATP-binding cassette transporter gene conferring resistance to the diterpenoid sclareol is regulated by multiple defense signaling pathways in *Arabidopsis*. Plant Physiol 133(3):1272–1284. doi:10.1104/pp. 103.024182

Cannon SB (2013) The model legume genomes. Methods Mol Biol 1069:1–14. doi:10.1007/978-1-62703-613-9_1

Choi H, Jin JY, Choi S, Hwang JU, Kim YY et al (2011) An ABCG/WBC-type ABC transporter is essential for transport of sporopollenin precursors for exine formation in developing pollen. Plant J 65(2):181–193. doi:10.1111/j.1365-313X.2010.04412.x

Cock JM, Sterck L, Rouze P, Scornet D, Allen AE et al (2010) The Ectocarpus genome and the independent evolution of multicellularity in brown algae. Nature 465(7298):617–621. doi:10.1038/nature09016

Crouzet J, Trombik T, Fraysse AS, Boutry M (2006) Organization and function of the plant pleiotropic drug resistance ABC transporter family. FEBS Lett 580(4):1123–1130

Crouzet J, Roland J, Peeters E, Trombik T, Ducos E et al (2013) NtPDR1, a plasma membrane ABC transporter from *Nicotiana tabacum*, is involved in diterpene transport. Plant Mol Biol 82(1–2):181–192. doi:10.1007/s11103-013-0053-0

Czaja LF, Hogekamp C, Lamm P, Maillet F, Martinez EA et al (2012) Transcriptional responses toward diffusible signals from symbiotic microbes reveal *MtNFP*- and *MtDMI3*-dependent reprogramming of host gene expression by arbuscular mycorrhizal fungal lipochitooligosaccharides. Plant Physiol 159(4):1671–1685. doi:10.1104/pp. 112.195990

Damiani I, Baldacci-Cresp F, Hopkins J, Andrio E, Balzergue S et al (2012) Plant genes involved in harbouring symbiotic rhizobia or pathogenic nematodes. New Phytol 194(2):511–522. doi:10.1111/j.1469-8137.2011.04046.x

Dangl JL, Jones JD (2001) Plant pathogens and integrated defence responses to infection. Nature 411(6839):826–833. doi:10.1038/35081161

Davis AS, Hill JD, Chase CA, Johanns AM, Liebman M (2012) Increasing cropping system diversity balances productivity, profitability and environmental health. PloS One 7(10):e47149. doi:10.1371/journal.pone.0047149

Dharmatilake AJ, Bauer WD (1992) Chemotaxis of *Rhizobium meliloti* towards Nodulation Gene-Inducing Compounds from Alfalfa roots. Appl Environ Microbiol 58(4):1153–1158

Dixon RA, Sumner LW (2003) Legume natural products: understanding and manipulating complex pathways for human and animal health. Plant Physiol 131(3):878–885. doi:10.1104/pp.102.017319

Dor E, Joel DM, Kapulnik Y, Koltai H, Hershenhorn J (2011) The synthetic strigolactone GR24 influences the growth pattern of phytopathogenic fungi. Planta 234(2):419–427. doi:10.1007/s00425-011-1452-6

Dou XY, Yang KZ, Zhang Y, Wang W, Liu XL et al (2011) WBC27, an adenosine tri-phosphate-binding cassette protein, controls pollen wall formation and patterning in Arabidopsis. J Integr Plant Biol 53(1):74–88. doi:10.1111/j.1744-7909.2010.01010.x

Eichhorn H, Klinghammer M, Becht P, Tenhaken R (2006) Isolation of a novel ABC-transporter gene from soybean induced by salicylic acid. J Exp Bot 57(10):2193–2201. doi:10.1093/jxb/erj179

Farag MA, Huhman DV, Dixon RA, Sumner LW (2008) Metabolomics reveals novel pathways and differential mechanistic and elicitor-specific responses in phenylpropanoid and isoflavonoid biosynthesis in *Medicago truncatula* cell cultures. Plant Physiol 146(2):387–402. doi:10.1104/pp. 107.108431

Gaude N, Bortfeld S, Duensing N, Lohse M, Krajinski F (2012) Arbuscule-containing and non-colonized cortical cells of mycorrhizal roots undergo extensive and specific reprogramming during arbuscular mycorrhizal development. Plant J 69(3):510–528. doi:10.1111/j.1365-313X.2011.04810.x

Grec S, Vanham D, de Ribaucourt JC, Purnelle B, Boutry M (2003) Identification of regulatory sequence elements within the transcription promoter region of NpABC1, a gene encoding a plant ABC transporter induced by diterpenes. Plant J 35(2):237–250

Gutjahr C, Novero M, Guether M, Montanari O, Udvardi M et al (2009) Presymbiotic factors released by the arbuscular mycorrhizal fungus Gigaspora margarita induce starch accumulation in *Lotus japonicus* roots. New Phytol 183(1):53–61. doi:10.1111/j.1469-8137.2009.02871.x

Gutjahr C, Radovanovic D, Geoffroy J, Zhang Q, Siegler H et al (2012) The half-size ABC transporters STR1 and STR2 are indispensable for mycorrhizal arbuscule formation in rice. Plant J 69(5):906–920. doi:10.1111/j.1365-313X.2011.04842.x

Hartmann K, Peiter E, Koch K, Schubert S, Schreiber L (2002) Chemical composition and ultrastructure of broad bean (*Vicia faba* L.) nodule endodermis in comparison to the root endodermis. Planta 215(1):14–25. doi:10.1007/s00425-001-0715-z

Hassan S, Mathesius U (2012) The role of flavonoids in root-rhizosphere signalling: opportunities and challenges for improving plant-microbe interactions. J Exp Bot 63(9):3429–3444. doi:10.1093/jxb/err430

Hogekamp C, Arndt D, Pereira PA, Becker JD, Hohnjec N et al (2011) Laser microdissection unravels cell-type-specific transcription in arbuscular mycorrhizal roots, including CAAT-box transcription factor gene expression correlating with fungal contact and spread. Plant Physiol 157(4):2023–2043. doi:10.1104/pp. 111.186635

Jasinski M, Stukkens Y, Degand H, Purnelle B, Marchand-Brynaert J et al (2001) A plant plasma membrane ATP binding cassette-type transporter is involved in antifungal terpenoid secretion. Plant Cell 13(5):1095–1107

Jasinski M, Banasiak J, Radom M, Kalitkiewicz A, Figlerowicz M (2009) Full-size ABC transporters from the ABCG subfamily in *Medicago truncatula*. Mol Plant-Microbe Interact 22(8):921–931. doi:10.1094/MPMI-22-8-0921

Jaulneau V, Cazaux M, Wong Sak Hoi J, Fournier S, Esquerre-Tugaye MT et al (2010) Host and nonhost resistance in Medicago-Colletotrichum interactions. Mol Plant-Microbe Interact 23(9):1107–1117. doi:10.1094/MPMI-23-9-1107

Jones JD, Dangl JL (2006) The plant immune system. Nature 444(7117):323–329. doi:10.1038/nature05286

Kang J, Hwang JU, Lee M, Kim YY, Assmann SM et al (2010) PDR-type ABC transporter mediates cellular uptake of the phytohormone abscisic acid. Proc Natl Acad Sci U S A 107(5):2355–2360. doi:10.1073/pnas.0909222107

Kang J, Park J, Choi H, Burla B, Kretzschmar T et al. (2011) Plant ABC Transporters. The Arabidopsis book/American Society of Plant Biologists 9:e0153. doi:10.1199/tab.0153

Kistner C, Parniske M (2002) Evolution of signal transduction in intracellular symbiosis. Trends Plant Sci 7(11):511–518

Kobae Y, Sekino T, Yoshioka H, Nakagawa T, Martinoia E et al (2006) Loss of AtPDR8, a plasma membrane ABC transporter of Arabidopsis thaliana, causes hypersensitive cell death upon pathogen infection. Plant Cell Physiol 47(3):309–318. doi:10.1093/pcp/pcj001

Kosslak RM, Bookland R, Barkei J, Paaren HE, Appelbaum ER (1987) Induction of Bradyrhizobium japonicum common nod genes by isoflavones isolated from Glycine max. Proc Natl Acad Sci U S A 84(21):7428–7432

Kouchi H, Shimomura K, Hata S, Hirota A, Wu GJ et al (2004) Large-scale analysis of gene expression profiles during early stages of root nodule formation in a model legume, *Lotus japonicus*. DNA Res 11(4):263–274

Krattinger SG, Lagudah ES, Spielmeyer W, Singh RP, Huerta-Espino J et al (2009) A putative ABC transporter confers durable resistance to multiple fungal pathogens in wheat. Science 323(5919):1360–1363. doi:10.1126/science.1166453

Kretzschmar T, Kohlen W, Sasse J, Borghi L, Schlegel M et al (2012) A petunia ABC protein controls strigolactone-dependent symbiotic signalling and branching. Nature 483(7389): 341–344. doi:10.1038/nature10873

Kuromori T, Miyaji T, Yabuuchi H, Shimizu H, Sugimoto E et al (2010) ABC transporter AtABCG25 is involved in abscisic acid transport and responses. Proc Natl Acad Sci U S A 107(5):2361–2366. doi:10.1073/pnas.0912516107

Limpens E, Moling S, Hooiveld G, Pereira PA, Bisseling T et al. (2013) Cell- and tissue-specific transcriptome analyses of *Medicago truncatula* root nodules. PloS one 8(5):e64377. doi:10.1371/journal.pone.0064377

Lipka U, Fuchs R, Lipka V (2008) Arabidopsis non-host resistance to powdery mildews. Curr Opini Plant Biol 11(4):404–411. doi:10.1016/j.pbi.2008.04.004

Liu J, Blaylock LA, Endre G, Cho J, Town CD et al (2003) Transcript profiling coupled with spatial expression analyses reveals genes involved in distinct developmental stages of an arbuscular mycorrhizal symbiosis. Plant Cell 15(9):2106–2123

Lozovaya VV, Lygin AV, Zernova OV, Li S, Hartman GL et al (2004) Isoflavonoid accumulation in soybean hairy roots upon treatment with *Fusarium solani*. Plant Physiology Biochem 42(7–8):671–679. doi:10.1016/j.plaphy.2004.06.007

McFarlane HE, Shin JJ, Bird DA, Samuels AL (2010) Arabidopsis ABCG transporters, which are required for export of diverse cuticular lipids, dimerize in different combinations. Plant Cell 22(9):3066–3075. doi:10.1105/tpc.110.077974

Moons A (2008) Transcriptional profiling of the PDR gene family in rice roots in response to plant growth regulators, redox perturbations and weak organic acid stresses. Planta 229(1):53–71. doi:10.1007/s00425-008-0810-5

Naoumkina M, Farag MA, Sumner LW, Tang Y, Liu CJ et al (2007) Different mechanisms for phytoalexin induction by pathogen and wound signals in *Medicago truncatula*. Proc Natl Acad Sci U S A 104(46):17909–17915. doi:10.1073/pnas.0708697104

Oldroyd GE (2013) Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants. Nat Rev Microbio 11(4):252–263. doi:10.1038/nrmicro2990

Oldroyd GE, Downie JA (2008) Coordinating nodule morphogenesis with rhizobial infection in legumes. Annu Rev Plant Biol 59:519–546. doi:10.1146/annurev.arplant.59.032607.092839

Oldroyd GE, Murray JD, Poole PS, Downie JA (2011) The rules of engagement in the legume-rhizobial symbiosis. Annu Rev Genet 45:119–144. doi:10.1146/annurev-genet-110410-132549

Ortu G, Balestrini R, Pereira PA, Becker JD, Kuster H et al (2012) Plant genes related to gibberellin biosynthesis and signaling are differentially regulated during the early stages of AM fungal interactions. Mol Plant 5(4):951–954. doi:10.1093/mp/sss027

Paape T, Zhou P, Branca A, Briskine R, Young N et al (2012) Fine-scale population recombination rates, hotspots, and correlates of recombination in the Medicago truncatula genome. Genome Biol Evol 4(5):726–737. doi:10.1093/gbe/evs046

Panikashvili D, Savaldi-Goldstein S, Mandel T, Yifhar T, Franke RB et al (2007) The Arabidopsis DESPERADO/AtWBC11 transporter is required for cutin and wax secretion. Plant Physiol 145(4):1345–1360. doi:10.1104/pp. 107.105676

Parniske M (2008) Arbuscular mycorrhiza: the mother of plant root endosymbioses. Nat Rev Microbiol 6(10):763–775. doi:10.1038/nrmicro1987

Pieterse CM, Leon-Reyes A, Van der Ent S, Van Wees SC (2009) Networking by small-molecule hormones in plant immunity. Nat Chem Biol 5(5):308–316. doi:10.1038/nchembio.164

Prell J, Poole P (2006) Metabolic changes of rhizobia in legume nodules. Trends Microbiol 14(4): 161–168. doi:10.1016/j.tim.2006.02.005

Quilichini TD, Friedmann MC, Samuels AL, Douglas CJ (2010) ATP-binding cassette transporter G26 is required for male fertility and pollen exine formation in Arabidopsis. Plant Physiol 154(2):678–690. doi:10.1104/pp. 110.161968

Rea PA (2007) Plant ATP-binding cassette transporters. Annu Rev Plant Biol 58:347–375. doi:10.1146/annurev.arplant.57.032905.105406

Sasabe M, Toyoda K, Shiraishi T, Inagaki Y, Ichinose Y (2002) cDNA cloning and characterization of tobacco ABC transporter: NtPDR1 is a novel elicitor-responsive gene. FEBS Lett 518(1–3):164–168

Schmutz J, Cannon SB, Schlueter J, Ma J, Mitros T et al (2010) Genome sequence of the palaeopolyploid soybean. Nature 463(7278):178–183. doi:10.1038/nature08670

Seo S, Gomi K, Kaku H, Abe H, Seto H et al (2012) Identification of natural diterpenes that inhibit bacterial wilt disease in tobacco, tomato and Arabidopsis. Plant Cell Physiol 53(8):1432–1444. doi:10.1093/pcp/pcs085

Singh S, Parniske M (2012) Activation of calcium- and calmodulin-dependent protein kinase (CCaMK), the central regulator of plant root endosymbiosis. Curr Opin Plant Biol 15(4): 444–453. doi:10.1016/j.pbi.2012.04.002

Smit G, Puvanesarajah V, Carlson RW, Barbour WM, Stacey G (1992) *Bradyrhizobium japonicum nodD1* can be specifically induced by soybean flavonoids that do not induce the *nodYABCSUIJ* operon. J Biol Chem 267(1):310–318

Stein M, Dittgen J, Sanchez-Rodriguez C, Hou BH, Molina A et al (2006) *Arabidopsis* PEN3/PDR8, an ATP binding cassette transporter, contributes to nonhost resistance to inappropriate pathogens that enter by direct penetration. Plant cell 18(3):731–746. doi:10.1105/tpc.105.038372

Stukkens Y, Bultreys A, Grec S, Trombik T, Vanham D et al (2005) NpPDR1, a pleiotropic drug resistance-type ATP-binding cassette transporter from *Nicotiana plumbaginifolia*, plays a major role in plant pathogen defense. Plant Physiol 139(1):341–352. doi:10.1104/pp. 105.062372

Sugiyama A, Shitan N, Sato S, Nakamura Y, Tabata S et al (2006) Genome-wide analysis of ATP-binding cassette (ABC) proteins in a model legume plant, *Lotus japonicus*: comparison with Arabidopsis ABC protein family. DNA Res 13(5):205–228. doi:10.1093/dnares/dsl013

Sugiyama A, Shitan N, Yazaki K (2007) Involvement of a soybean ATP-binding cassette-type transporter in the secretion of genistein, a signal flavonoid in legume-*Rhizobium* symbiosis. Plant Physiol 144(4):2000–2008. doi:10.1104/pp. 107.096727

Sugiyama A, Shitan N, Yazaki K (2008) Signaling from soybean roots to rhizobium: an ATP-binding cassette-type transporter mediates genistein secretion. Plant Signal Behav 3(1): 38–40

Tyler BM, Tripathy S, Zhang X, Dehal P, Jiang RH et al (2006) Phytophthora genome sequences uncover evolutionary origins and mechanisms of pathogenesis. Science 313(5791):1261–1266. doi:10.1126/science.1128796

Underwood W, Somerville SC (2013) Perception of conserved pathogen elicitors at the plasma membrane leads to relocalization of the Arabidopsis PEN3 transporter. Proc Natl Acad Sci U S A 110(30):12492–12497. doi:10.1073/pnas.1218701110

van den Brule S, Muller A, Fleming AJ, Smart CC (2002) The ABC transporter SpTUR2 confers resistance to the antifungal diterpene sclareol. Plant J: Cell Mol Biol 30(6):649–662

Verrier PJ, Bird D, Burla B, Dassa E, Forestier C et al (2008) Plant ABC proteins–a unified nomenclature and updated inventory. Trends Plant Sci 13(4):151–159. doi:10.1016/j.tplants.2008.02.001

Xin XF, Nomura K, Underwood W, He SY (2013) Induction and suppression of PEN3 focal accumulation during Pseudomonas syringae pv. tomato DC3000 infection of Arabidopsis. Mol Plant Microbe Interact 26(8):861–867. doi:10.1094/MPMI-11-12-0262-R

Young ND, Bharti AK (2012) Genome-enabled insights into legume biology. Annu Rev Plant Biol 63:283–305. doi:10.1146/annurev-arplant-042110-103754

Young ND, Debelle F, Oldroyd GE, Geurts R, Cannon SB et al (2011) The *Medicago* genome provides insight into the evolution of rhizobial symbioses. Nature 480(7378):520–524. doi:10.1038/nature10625

Zhang Q, Blaylock LA, Harrison MJ (2010) Two *Medicago truncatula* half-ABC transporters are essential for arbuscule development in arbuscular mycorrhizal symbiosis. Plant Cell 22(5):1483–1497. doi:10.1105/tpc.110.074955

Zhang R, Huang J, Zhu J, Xie X, Tang Q et al (2013) Isolation and characterization of a novel PDR-type ABC transporter gene PgPDR3 from Panax ginseng C.A. Meyer induced by methyl jasmonate. Mol Biol Rep 40(11):6195–6204. doi:10.1007/s11033-013-2731-z

Zipfel C (2008) Pattern-recognition receptors in plant innate immunity. Curr Opin Immunol 20(1):10–16. doi:10.1016/j.coi.2007.11.003

Zipfel C, Robatzek S (2010) Pathogen-associated molecular pattern-triggered immunity: veni, vidi. . .? Plant Physiol 154(2):551–554. doi:10.1104/pp. 110.161547

ABC Proteins and Other Transporters in *Lotus japonicus* and *Glycine max*

Kojiro Takanashi and Kazufumi Yazaki

Abstract Legume plants establish a symbiosis with nitrogen-fixing bacteria called rhizobia to gain nitrogen nutrients directly from atmospheric N_2 via symbiotic nitrogen fixation. This process annually provides approximately 33–46 million tons of fixed nitrogen in agricultural systems. For the establishment and maintenance of symbiotic nitrogen fixation a number of proteins, ions, and metabolites including fixed-nitrogen and photosynthetic products are exchanged between host plants and rhizobia across several membranes by a variety of transporters of both host plants and rhizobia. Many research groups have so far identified various enzymes and transporter proteins involved in the symbiosis over several decades. In this chapter we review the current knowledge on ABC proteins in legumes, especially related to symbiotic nitrogen fixation in determinate nodules representatively formed in *Glycine max* and *Lotus japonicus*. We also give an overview of other plant transporters involved in symbiotic nitrogen fixation in these legume plants.

1 Introduction

Legume plants develop a symbiotic organ called a nodule with nitrogen-fixing rhizobia. In nodules, atmospheric N_2 is converted to nitrogen nutrients via symbiotic nitrogen fixation (SNF), which provides in agricultural systems approximately 33–46 million tons of fixed nitrogen annually (Herridge et al. 2008). SNF occurs in nodules, which develop from dedifferentiated root cells after infection by species-specific rhizobia, where orchestrated gene expressions and exchange of

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chemical signals are involved (Murray 2011; Oldroyd 2013). Rhizobia infected into nodule cells via endocytosis transform into bacteroids surrounded by an inside-out plant plasma membrane (PM), called the symbiosome membrane (SM), resulting in the creation of a novel organelle termed a symbiosome (Goodchild and Bergersen 1966; Roth and Stacey 1989). In symbiosomes, atmospheric N_2 is reduced into ammonia that is supplied to plants, and in turn, photosynthates are provided from plants to bacteroids. The exchange of nitrogen and carbon metabolites is mediated by crossing at least three membranes; the PM of infected cells, the SM, and the bacteroid membrane, from the nodule vascular bundle to the bacteroid. This suggests many membrane transport events are involved in SNF to exchange signals and nutrients between a host legume and rhizobia. Indeed, a number of transcriptome, proteome, and metabolome analyses have been done in both plants and rhizobia and have revealed that various membrane transport molecules seem to be relevant for SNF (Udvardi and Poole 2013).

There are two types of root nodules in legume plants and both have been respectively investigated in detail (Sprent 2007). One of them, indeterminate nodules formed on legumes such as *Medicago truncatula* and *Pisum sativum* are initiated from the inner cortex, and the nodules have a persistent meristem that allows continuous growth and infection. The indeterminate nodules can be divided into four major zones, i.e., a persistent apical meristem (zone I), a bacterial infection zone (zone II), a nitrogen-fixing zone (zone III), and a senescence zone (zone IV) (Vasse et al. 1990). By contrast, the development of determinate nodules (e.g., in legumes like *Lotus japonicus* and *Glycine max*) begins from the cell divisions of the outer root cortex cell layer. These determinate nodules have a defined lifespan and lose their central meristem as well as the ability to be continuously infected upon maturation.

Hormones and proteins involved in the initiation, the development, and the maintenance of nodules are different between these two types of nodules (Mathesius 2008), implicating apparent differences in the regulation systems between these nodule types. To date, genome sequences of five legumes were published including *M. truncatula* with indeterminate nodules (Young et al. 2011), and two legumes forming determinate nodules, *L. japonicus* and *G. max* (Sato et al. 2008; Schmutz et al. 2010).

In this chapter we provide an overview of ABC proteins related to SNF in the determinate nodule-forming legumes, *L. japonicus* and *G. max*. First, we introduce a genome-wide analysis of ABC proteins in both *Lotus* and *Glycine* genomes, and then we discuss possible functions of individual ABC proteins. We also describe other transport systems, such as MATE (multidrug and toxic compound extrusion)-type transporters, which are relevant for SNF in legume plants.

2 ABC Proteins in the Genome of *L. japonicus* and *G. max*

Fabaceae is the third largest family of flowering plants, comprising more than 700 genera and 20,000 species, many of which have long been targets of breeding because of their agronomic and industrial importance. Among them, *L. japonicus* has been chosen as a representative model legume for genetic and physiological studies because of its short life cycle (2–3 months), self-fertility, and relatively small genome size ($n = 6$, 472 Mb). Prior to the report of *Lotus* genome structure and features (Sato et al. 2008), an inventory of *Lotus* ABC transporters was provided with coverage of 67 % (315 Mb) of the whole genome, to obtain a general feature of legume ABC transporters (Sugiyama et al. 2006). In this early study, because most of the detected open reading frames were fragmental, a domain-based clustering analysis was employed, in which the amino acid sequence of nucleotide binding domains (NBDs) and transmembrane domains (TMDs) instead of the entire proteins were used to cluster ABC protein fragments. At least 90 ABC transporter genes were found from the determined genome, in which a high redundancy of AtABCC14-like genes and AtABCG40-like genes were detected as a characteristic feature of *Lotus* ABC proteins (although originally 91 ABC genes were found, one gene in the SNC family was not counted in the present review according to the nomenclature proposed in 2008 (Verrier et al. 2008)). Similarly, the high redundancy of *AtABCG40*-like genes was also found in another model legume, *M. truncatula*, which develops indeterminate-type nodules (Jasinski et al. 2009).

Neither a physiological role nor the protein function of AtABCC14 has yet been reported in *Arabidopsis*, whereas *AtABCG40* has been identified as a gene induced by a fungal pathogen (*Alternaria brassicicola*) inoculation (Campbell et al. 2003). AtABCG40 was later reported as an uptake transporter of the sesquiterpene plant hormone, abscisic acid (Kang et al. 2010; see also chapter "ABC transporters and heavy metals"). Recently, its ortholog in *Petunia hybrida*, PhPDR1 has been suggested to function as a strigolactone exporter and to regulate the symbiosis of arbuscular mycorrhizae (Kretzschmar et al. 2012). In addition, a *Nicotiana* ortholog, NpPDR1, was reported to be involved in the secretion of sclareol, a diterpene phytoalexin (Gomez et al. 2009), and a suppression of *NpPDR1* increased the susceptibility to the plant pathogen, *Botrytis cinerea* (Stukkens et al. 2005). Another tobacco ortholog, NtPDR1, was also reported to transport several diterpenes including sclareol (Crouzet et al. 2013), suggesting that these ABCG members are involved in defense responses against both abiotic and biotic stresses, especially plant–microbe interaction (see also chapter "Plastidic ABC proteins"). In the *Lotus* genome most of the *AtABCG40*-like genes were upregulated in the root by inoculation with rhizobia, suggesting that this unique feature has an important role related to SNF (Sugiyama et al. 2006). One of those *AtABCG40*-like genes was designed as *LjABCG1*, which is described below.

Soybeans are one of the most important legumes as crops, representing 50 % of the global crop legume area and 68 % of global production. In 2010, the genome sequence of *G. max* var. Williams 82 was reported with the coverage of 85 %

Table 1 Comparison of the ABC protein in plants

	Genome (Mb)	ABCA	ABCB	ABCC	ABCD	ABCE	ABCF	ABCG	ABCI	Total	Reference
G. max	1,115	7 (6)	49 (9)	39	5 (1)	2	11	108 (73)	21	242	This review
L. japonicus	472	3 (1)	15 (3)	17	2 (1)	1	6	36 (24)	10	90	Sugiyama et al. (2006)
Arabidopsis	125	12 (11)	29 (7)	15	2 (1)	3	5	43 (28)	21	130	(Sanchez-Fernandez et al. 2001; Verrier et al. 2008)

Parentheses indicate the number of half-size ABC proteins for the subfamilies, in which both full- and half-size members are classified as one group

(950 Mb) of the predicted genome (1.115 Mb), which revealed a highly duplicated genome of the soybean, e.g., almost 75 % of genes were in multiple copies, due to genome duplication events that occurred twice 13 and 59 million years ago (Schmutz et al. 2010). To find the soybean-specific diversification in ABC transporters, a domain-based clustering analysis was performed with a gene data set ver. 1.09 (http://www.phytozome.net/soybean.php), from which 262 predicted genes were found to show high amino acid similarity with ABC proteins. Of those, 242 genes containing sequences of NBD and/or TMD were classified according to the nomenclature unified by Verrier et al. (Verrier et al. 2008) (Table 1, Fig. 1a–d). The total number is approximately double of those of *Lotus* and *Arabidopsis* (Sanchez-Fernandez et al. 2001; Sugiyama et al. 2006), which apparently reflects the larger genome size of *Glycine*. It is noteworthy that the high redundancy of AtABCG40-like genes, which was found in the *Lotus* and *Medicago* genomes, was also detected in the *Glycine* genome. Fourteen of 32 NBD1 sequences and nine of 35 NBD2s were exclusively similar to AtABCG38 and AtABCG40 (Fig. 1c). One full-size ABCG member in this clade, *GmPDR12*, was previously reported as a salicylic acid-induced gene in *G. max* var. Williams 82 cell suspension cultures (Eichhorn et al. 2006), and its expression was also induced by methyl jasmonate, suggesting that soybean ABC proteins in this clade are also involved in plant–microbe interaction as those of other plants. The higher redundancy of AtABCG40-like genes in the legume genome is presumed to reflect a variety of the legume–microorganism interaction, e.g., SNF and mycorrhizal symbiosis. The sequence of GmPDR12 was not found in this analysis, and the highest similarity with 98 % was found in a sequence of Glyma03g32520.1.

In the same ABCG subfamily, but in half-size members, 14 of 73 NBD genes showed a high similarity with AtABCG11 (Fig. 1d), which is responsible for wax load and thus, is proposed to transport wax components such as C29 alkanes and cutin monomers (Bird et al. 2007; Luo et al. 2007; Panikashvili et al. 2007; Ukitsu et al. 2007). Because this redundancy of AtABCG11-like genes was not detected in the *Lotus* genome, where putative ABCG NBDs were dispersed on the phylogenetic tree (Sugiyama et al. 2006), the redundancy of AtABCG11-like genes may not be related in SNF. A cluster of AtABCC14-like genes, another characteristic clade of high redundancy observed in the *Lotus* genome, was not found in this analysis with soybeans (Fig. 1b).

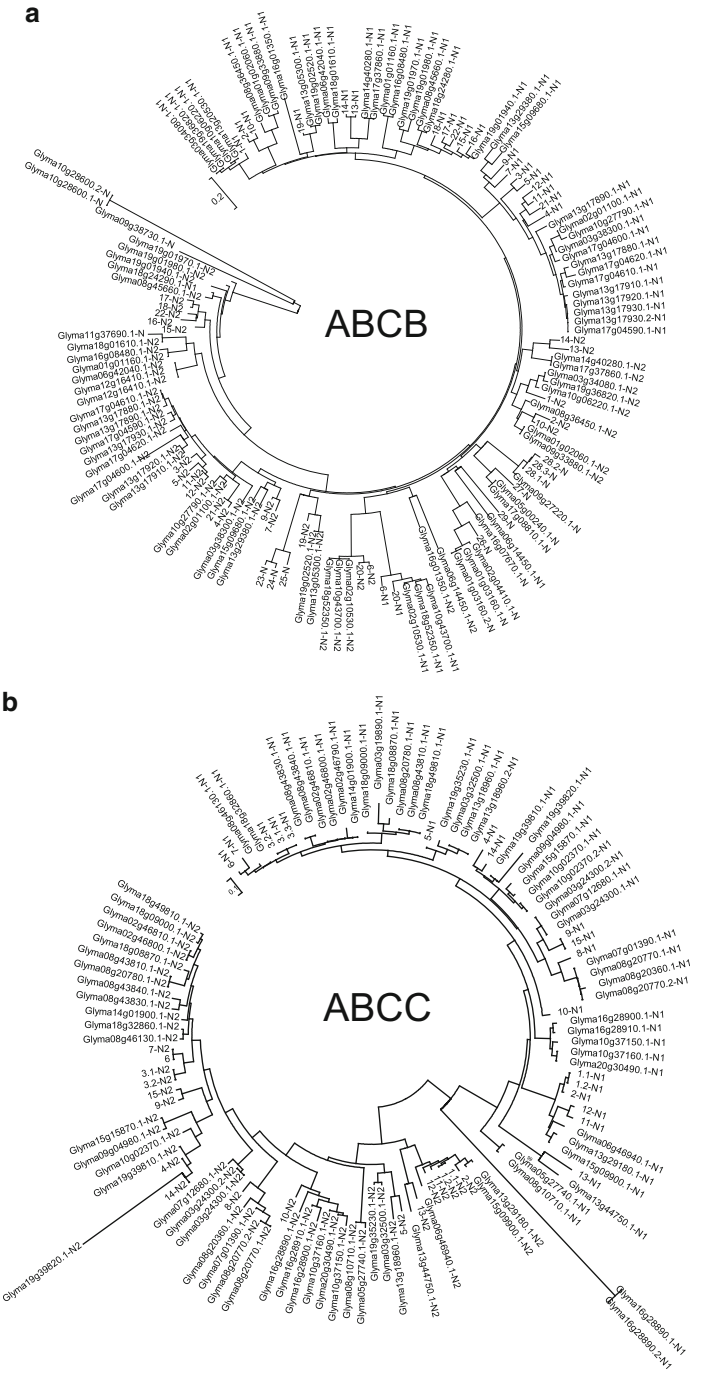

Fig. 1 (continued)

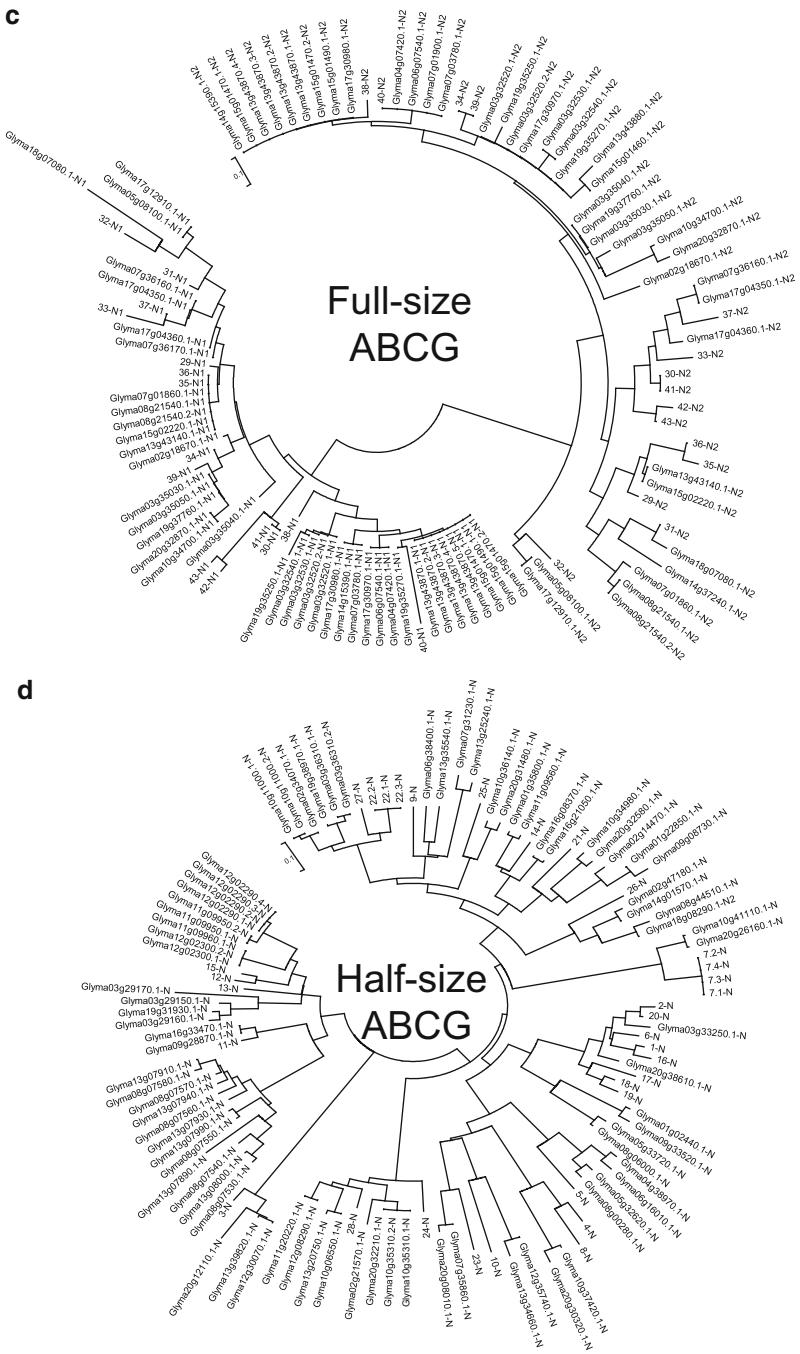

Fig. 1 Domain-based clustering analyses of (**a**) ABCB, (**b**) ABCC, (**c**) full-size ABCG, and (**d**) half-size ABCG in *G. max* and *Arabidopsis* genomes. Phylogenetic trees were generated with the amino acid sequences of NBDs using MEGA 5.0 software (Tamura et al. 2011). N1 and N2 represent NBD1 and NBD2, respectively. ABC members of *Arabidopsis* are represented as the number

3 Functions of ABC Proteins in Symbiosis with Rhizobia

3.1 ABC Proteins Mediate the Exchange of Signaling Molecules Between Legume and Rhizobia

Nodule development in the legume is initiated by an exchange of chemical signals between plant root cells and soil rhizobia, which is a determinant factor of species-specific symbiosis. In nitrogen deficient conditions, legume plants release particular flavonoids as the symbiosis signal into the root exudates, e.g., luteolin from alfalfa (*Medicago sativa*), 7,4′-dihydroxyflavone and geraldone from white clover (*Trifolium repens*), and daidzein and genistein from *G. max* (Djordjevic et al. 1987; Kosslak et al. 1987; Peters et al. 1986; Redmond et al. 1986). These flavonoids differ according to legume species, suggesting that a structurally different flavonoid determines species-specific interaction between plant and rhizobia, at least in part. The transport system of this flavonoid secretion was biochemically analyzed using membrane vesicles of soybean root and revealed that the transport characteristics are typical for those of ABC transporters (Sugiyama et al. 2007). Genistein was transported in an ATP-dependent manner, while its transport was inhibited by vanadate, a general inhibitor for ABC transporters and P-type ATPases. The genistein transport was not influenced by other inhibitors such as bafilomycin A1 and gramicidin D, which act as ionophores to destroy the electrochemical gradient across the membrane, on which secondary transport depends. Out of the ABC transporters, ABCG members were suggested particularly to mediate the genistein secretion from soybean root because no inhibitors of ABCB and ABCC affected genistein transport (Sugiyama et al. 2007). It is of interest that the glucoside of isoflavones genistin and daidzin (7-*O*-glycoside of genistein and daidzein, respectively) as well as their 6″-*O*-malonyled compounds (6″-*O*-malonylgenistin and 6″-*O*-malonyldaidzin) were not transported in an ATP-dependent manner. However, an abundance of malonylated and nonmalonylated genistin and daidzin were detected in a soybean root exudate (Pueppke et al. 1998). The existence of an apoplastic soybean β-glucosidase, GmICHG, which hydrolyzes the β-glucosidic linkage of glucosides suggests the involvement of other transport systems of these flavonoids across PM (Suzuki et al. 2006).

A MATE-type transporter family that constitutes one of the largest transporter families, e.g., 56 genes in *Arabidopsis* genome and at least 100 members in *G. max*, is one candidate responsible for the secretion of those hydrophilic flavonoid glucosides. Because the MATE family transports a broad range of substrates, some of which, e.g., alkaloid and phenylpropanoid, overlap with those of the ABC protein family (Alejandro et al. 2012; Otani et al. 2005; Shitan et al. 2003; Tsuyama et al. 2013), the existence of coordinated regulation of metabolite dynamics by ABC and MATE transporters is suggested. Several MATE transporters have also been reported to mediate the flavonoid accumulation into the vacuole. AtTT12 from *Arabidopsis* and MtMATE1 of *M. truncatula* transport cyanidin 3-*O*-glucoside and

epicatechin 3′-*O*-glucoside in yeast (Marinova et al. 2007; Zhao and Dixon 2009), and both are the precursors of proanthocyanidins. In the grapevine, two MATE members, VvAM1 and VvAM3, accumulate acylated cyanidin 3-*O*-glucoside into the vacuole (Gomez et al. 2009). By contrast, MATE transporters involved in the flavonoid efflux at the PM have not been reported so far; however, recent study by Tsuyama et al. suggested the existence of an H^+-antiporter-coupled vesicle transport system into an apoplast (Tsuyama et al. 2013). This type of transport system might function in the secretion of flavonoid glucoside in legume roots. Some genes encoding AtTT12-like MATE transporters are expressed highly in *Glycine* roots (Libault et al. 2010); thus, it will be interesting to determine their membrane localization and transport substrate in detail.

In response to plant-secreted flavonoids, which bind to the NodD receptor, rhizobia produce and release Nod factors that are lipochitooligosaccharide derivatives containing various species-specific modifications especially in the side chain, which are essential for the specific host–rhizobia interactions (Egelhoff and Long 1985; Fisher and Long 1992; Mulligan and Long 1985; Rossen et al. 1985). The secretion of Nod factors from rhizobia is mediated by a bacterial type ABC transporter that consists of the products of *nodI* and *nodJ* genes (McKay and Djordjevic 1993; Spaink et al. 1995), both of which construct an operon together with the genes of Nod factor biosynthesis, *nodA*, *nodB*, and *nodC* (Bulawa and Wasco 1991; Lerouge et al. 1990; Schmidt et al. 1988). Because a low level of secretion of Nod factor was detected in the *nodJ* mutant, some involvement of other transporters, e.g., nolFGHI, is also suggested (Saier et al. 1994).

3.2 *ABC Proteins Functions in the Mature Phase of Determinate Nodules*

After perception of Nod factors by plant root hair cells, a curling of the root hair cells is triggered, and then rhizobia can penetrate into the cortical cells through elongation of an infection thread, which successively causes cortical cell division. During nodule developments, both plant cells and rhizobia differentiate concertedly, resulting in the formation of both infected and uninfected plant cells in a pavement pattern inside the nodule. Then, fixed nitrogen was supplied to the plant from the rhizobia in SM in infected cells, and the plant provided reduced carbon and all other nutrients necessary for symbiotic-bacteria, in turn (Oldroyd et al. 2011; Udvardi and Poole 2013).

To understand the metabolite dynamics in *Lotus* nodules, tissue-specific transcriptome analysis has been recently performed using laser microdissection (Takanashi et al. 2012b). Three tissues, i.e., infection zone (IFZ), nodule parenchyma (NP), and nodule cortex (NC), were isolated from the sections of matured nodules (Fig. 2a–e), and each expressing gene was identified by microarray analyses. Totally, 1,425 genes were found in the nodule, of which approximately 65 %

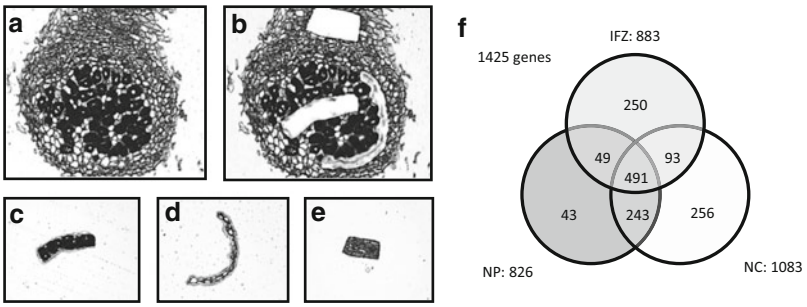

Fig. 2 Isolation of three tissues from the cross-sections of *L. japonicus* nodules using laser microdissection. Cross-section of a nodule (**a**) before and (**b**) after LM. (**c**) Infection zone (IFZ), (**d**) nodule parenchyma (NP), and (**e**) nodule inner cortex (NC). (**f**) The number of genes expressing in IFZ, NP, and NC. (Figures were taken from Takanashi et al. 2012b)

genes were expressed in a tissue-specific manner (Fig. 2f). From this analysis, nine ABC proteins were detected; five ABC genes showed IFZ-specific expression and two genes were NC-specific, whereas the other four ABC proteins were expressed ubiquitously in nodules, some of which were reported previously as genes induced in SNF (Kouchi et al. 2004; Sugiyama et al. 2006).

One of the IFZ-specific ABC proteins was LjABCB1, which has a high amino acid similarity with AtABCB4 (67 %) and AtABCB21 (68 %), both of which were reported as auxin transporters in *Arabidopsis* root (Cho et al. 2007, 2012; Kamimoto et al. 2012; Lewis et al. 2007a, b; Santelia et al. 2005; Terasaka et al. 2005). Expression of *LjABCB1* was induced only in the uninfected cells adjacent to the infected cells in the late nodulation stage (Takanashi et al. 2012a). The same expression was previously observed in a GUS-trapped line, FATA MORGANA (Buzas et al. 2005). When expressed in yeast, LjABCB1 showed IAA efflux activity, but the transport activity was much less than in those of AtABCB21 (Kamimoto et al. 2012; Takanashi et al. 2012a). During nodule development, auxin is first accumulated in the proliferating root cortical cells, where rhizobia are infected. Then auxin accumulation is detected in the dividing cortical cells of the nodule primordia, and finally at the vascular bundle in the matured nodules (Pacios-Bras et al. 2003; Suzaki et al. 2012; Takanashi et al. 2011). Upon the treatment of synthetic inhibitors of auxin transport during nodule development, the formation of both the nodule vascular bundle and lenticels were clearly inhibited (Takanashi et al. 2011). Because RNAi transformants of *LjABCB1* showed no visible phenotype difference, the physiological function of the LjABCB1 is still to be investigated. However, it is noteworthy that *LjABCB1* expression was highly induced in Fix^- mutant nodules such as *fen1* and *sen1*, which have a mutation in a homocitrate synthase and vacuolar iron transport-like gene, respectively (Hakoyama et al. 2009; Suganuma et al. 2003). Considering the higher expression of *LjABCB1* in Fix^- mutants with the restricted expression of *LjABCB1* in uninfected cells adjacent to infected cells, it is suggested that LjABCB1 is involved in the maturation and maintenance of infected cells. To clarify the

physiological function of LjABCB1, it will be necessary to visualize local auxin dynamics with higher resolution during nodule development.

LjABCB1, a member belonging to the *Lotus*-specific ABCG clade as described above, was also found in the tissue-specific transcriptome analysis as an NC-specific ABC gene (Takanashi et al. 2012b). This full-size ABCG transporter was first reported as a transiently induced gene at 4–7days after inoculation of rhizobia from time-course transcriptome analysis (Kouchi et al. 2004). *LjABCG1* was expressed in the primary root tip, steles of lateral roots, the root–nodule junction, and the nodule parenchyma (Sugiyama and Yazaki, unpublished result). The transport assay revealed that LjABCG1 does not transport sclareol (Sugiyama and Yazaki, unpublished result), the substrates of LjABCG1 orthologs in *Nicotiana* (Jasinski et al. 2001). Recently it has been reported that the silencing of *MtABCG10*, an ortholog of *LjABCG1*in *M. truncatula*, decreased flavonoid contents both in root tissues and root exudate (Banasiak et al. 2013; see also chapter "Defence, symbiosis and ABCG transporters"), suggesting that flavonoids are one of the candidates of transported substrates for LjABCG1. The fact that *LjABCG1* and flavonoid biosynthesis genes showed similar spatiotemporal expression pattern also support this idea (Kouchi et al. 2004; Takanashi et al. 2012b).

The rhizobia ABC transporter also functions in the matured nodule to promote SNF. The ModABC transporter of *B. japonicum* mediates high-affinity molybdate uptake, and *mod* mutants reduced the nitrogenase activity in symbiosis with soybeans under a Mo-deficient condition (Delgado et al. 2006). This reduction of Mo content became severer when sulfate was supplied to the growth medium of soybean symbiont, suggesting the existence of a Mo uptake system mediated by a sulfate transporter. Mo is an element absolutely necessary for SNF because nitrogenase, the key enzyme for nitrogen fixation, is a molybdenum–iron requiring protein, which has a heterometal center (FeMo cofactor) as the catalytic site (Dixon and Kahn 2004). The legume protein responsible for Mo transport on either PM or SM in nodules has not yet been identified. Some metal transporters found in IFZ from tissue-specific transcriptome analysis probably mediate Mo uptake into the symbiosome.

4 Other Transporters Functioning in *Lotus* and *Glycine* Nodules

In addition to ABC transporters, a number of transporter genes are expected to function in matured nodules. In fact, a MATE-type protein, LjMATE1, was found from the tissue-specific transcriptome analysis to be expressed in IFZ (Takanashi et al. 2012b). LjMATE1 transports citrate when expressed in *Xenopus* oocyte, and when expression of *LjMATE1* was silenced in *L. japonicus*, nitrogenase activity and leghemoglobin expression were significantly reduced compared to control plants, apparently due to less iron accumulation in IFZ. By contrast, increased iron

deposition was observed in the nodule-root junction and the nodule vascular bundle in *LjMATE1*-silencing plants, suggesting that the efflux of citrate into the apoplast by LjMATE1 is important for iron translocation from the vascular bundle to IFZ (Takanashi et al. 2013). To confirm this idea, the membrane localization of LjMATE1 is to be determined. Iron translocation is of a special importance in nodules, especially in infected cells, because nitrogenase requires iron for an active site of MoFe protein, and also for another component, the iron protein that functions as an ATP-dependent electron donor to MoFe protein (Dixon and Kahn 2004). Iron, as well as nitrogenase, is essential for other proteins such as ferredoxin, cytochrome, and leghemoglobin for their activities (Appleby 1984; Dixon and Kahn 2004). In fact, approximately 44 % of the iron within soybean plants is estimated to be in the nodule (Burton et al. 1998). To date, only one iron transporter has been reported in relation to SNF. GmDMT1, a NRAMP (natural resistance-associated macrophage protein) transporter of *G. max*, was identified to transport ferrous iron at SM into the symbiosome (Kaiser et al. 2003). For more detail, mechanisms of iron uptake and translocation in legume nodules have been summarized in a recent review (Brear et al. 2013).

Transport mechanisms of primary metabolites and ions across SM were studied biochemically using isolated symbiosome from soybeans in the 1990s (LeVier et al. 1996; Tyerman et al. 1995; Udvardi and Day 1989; Udvardi et al. 1988, 1990, 1991), and to identify the proteins involved in those, the proteome analyses of SM were performed in several legume species (Catalano et al. 2004; Panter et al. 2000; Saalbach et al. 2002; Wienkoop and Saalbach 2003), which indicated that a variety of transporters function on SM, including ABC proteins. To the date no ABC transporters on SM have been investigated; however, several transporters have been reported with forward and reverse genetic approaches, e.g., a sulfate transporter, LjSST1, from *L. japonicus* (Krusell et al. 2005), a zinc transporter (GmZIP1) from soybeans (Moreau et al. 2002), and anion transporters of both *Lotus* (LjN70) and the soybean (GmN70) (Szczyglowski et al. 1998; Vincill et al. 2005). Very recently, the membrane localization of a vacuolar iron transporter LjSEN1 was determined to be at the SM (T. Hakoyama, personal communication). Because mutation in the *LjSEN1* gene causes a severe fix- phenotype, the characterization of its transport substrate is of high interest (Hakoyama et al. 2012). Although the detailed membrane localization has not been determined, an ammonium transporter, LjAMT2;1 (Simon-Rosin et al. 2003), and a K^+ transporter LjKUP (Desbrosses et al. 2004) are characterized to function also in the infected cells of *L. japonicus*. As a transporter potentially involved in the carbon supply for bacteroids, LjSUT4 was also reported, which belongs a type-III sucrose transporter family localized at the tonoplast of *L. japonicus* nodule, and takes up a range of glucosides including sucrose into cytosol according to the proton gradient (Flemetakis et al. 2003; Reinders et al. 2008).

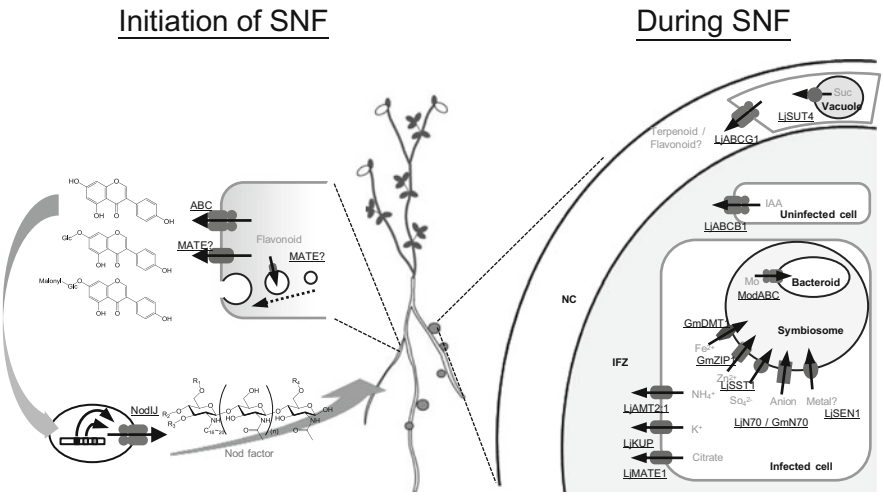

Fig. 3 A proposed model of ABC proteins and other transporters functioning in SNF

5 Conclusions

Because of the agronomical importance of legume crops, investigation of SNF mechanisms in legumes has been of great interest for many decades. In particular, the transport of metabolites between legume plants and rhizobia has been one of the focuses of intense research. This chapter focused on such transporter molecules, mostly featuring ABC proteins, involved in SNF mainly in *G. max* and *L. japonicus* as representatives of determinate nodule-forming legumes (Fig. 3).

Although a variety of metabolites and ions are exchanged during the development and function of SNF, only a few ABC proteins have been analyzed to date. This seems to be due to the difficulties in supposition and identification of the transport substrates of ABC proteins because of their restricted substrate specificities for their own metabolites and also of the low correlation between the amino acid sequence similarity and the type of native substrates. Moreover, recent work by Yu and De Luca (2013) revealed that the translocation of catharanthine, an intermediate in the biosynthesis of the monoterpene indole alkaloid, is also mediated by the ABC transporter in *Catharanthus roseus* (Yu and De Luca 2013), indicating the necessity of a comprehensive understanding of metabolite dynamics for transporter studies. Drawing the metabolic pathways in nodules with higher spatial and temporal resolutions using recently established systems, e.g., an imaging mass spectrometry technique (Yoshimura et al. 2012) and a real-time radioisotope imaging system (Kanno et al. 2012), are ways to understand metabolite dynamics in the nodules, which will facilitate the characterization of more transporters including ABC proteins functioning in SNF.

References

Alejandro S, Lee Y, Tohge T, Sudre D, Osorio S, Park J, Bovet L, Geldner N, Fernie AR, Martinoia E (2012) AtABCG29 is a monolignol transporter involved in lignin biosynthesis. Curr Biol 22:1207–1212

Appleby CA (1984) Leghemoglobin and rhizobium respiration. Annu Rev Plant Phys 35:443–478

Banasiak J, Biala W, Staszkow A, Swarcewicz B, Kepczynska E, Figlerowicz M, Jasinski M (2013) A *Medicago truncatula* ABC transporter belonging to subfamily G modulates the level of isoflavonoids. J Exp Bot 64:1005–1015

Bird D, Beisson F, Brigham A, Shin J, Greer S, Jetter R, Kunst L, Wu X, Yephremov A, Samuels L (2007) Characterization of Arabidopsis ABCG11/WBC11, an ATP binding cassette (ABC) transporter that is required for cuticular lipid secretion. Plant J 52:485–498

Brear EM, Day DA, Smith PM (2013) Iron: an essential micronutrient for the legume-rhizobium symbiosis. Front Plant Sci 4:359

Bulawa CE, Wasco W (1991) Chitin and nodulation. Nature 353:710

Burton JW, Harlow C, Theil EC (1998) Evidence for reutilization of nodule iron in soybean seed development. J Plant Nutr 21:913–927

Buzas DM, Lohar D, Sato S, Nakamura Y, Tabata S, Vickers CE, Stiller J, Gresshoff PM (2005) Promoter trapping in *Lotus japonicus* reveals novel root and nodule GUS expression domains. Plant Cell Physiol 46:1202–1212

Campbell EJ, Schenk PM, Kazan K, Penninckx IA, Anderson JP, Maclean DJ, Cammue BP, Ebert PR, Manners JM (2003) Pathogen-responsive expression of a putative ATP-binding cassette transporter gene conferring resistance to the diterpenoid sclareol is regulated by multiple defense signaling pathways in Arabidopsis. Plant Physiol 133:1272–1284

Catalano CM, Lane WS, Sherrier DJ (2004) Biochemical characterization of symbiosome membrane proteins from *Medicago truncatula* root nodules. Electrophoresis 25:519–531

Cho M, Lee SH, Cho HT (2007) P-glycoprotein 4 displays auxin efflux transporter-like action in *Arabidopsis* root hair cells and tobacco cells. Plant Cell 19:3930–3943

Cho M, Lee ZW, Cho HT (2012) ATP-binding cassette B4, an auxin-efflux transporter, stably associates with the plasma membrane and shows distinctive intracellular trafficking from that of PIN-FORMED proteins. Plant Physiol 159:642–654

Crouzet J, Roland J, Peeters E, Trombik T, Ducos E, Nader J, Boutry M (2013) NtPDR1, a plasma membrane ABC transporter from *Nicotiana tabacum*, is involved in diterpene transport. Plant Mol Biol 82:181–192

Delgado MJ, Tresierra-Ayala A, Talbi C, Bedmar EJ (2006) Functional characterization of the *Bradyrhizobium japonicum modA* and *modB* genes involved in molybdenum transport. Microbiology 152:199–207

Desbrosses G, Kopka C, Ott T, Udvardi MK (2004) *Lotus japonicus LjKUP* is induced late during nodule development and encodes a potassium transporter of the plasma membrane. Mol Plant Microbe Interact 17:789–797

Dixon R, Kahn D (2004) Genetic regulation of biological nitrogen fixation. Nat Rev Microbiol 2: 621–631

Djordjevic MA, Redmond JW, Batley M, Rolfe BG (1987) Clovers secrete specific phenolic compounds which either stimulate or repress nod gene expression in *Rhizobium trifolii*. EMBO J 6:1173–1179

Egelhoff TT, Long SR (1985) *Rhizobium meliloti* nodulation genes: identification of nodDABC gene products, purification of nodA protein, and expression of nodA in *Rhizobium meliloti*. J Bacteriol 164:591–599

Eichhorn H, Klinghammer M, Becht P, Tenhaken R (2006) Isolation of a novel ABC-transporter gene from soybean induced by salicylic acid. J Exp Bot 57:2193–2201

Fisher RF, Long SR (1992) Rhizobium–plant signal exchange. Nature 357:655–660

Flemetakis E, Dimou M, Cotzur D, Efrose RC, Aivalakis G, Colebatch G, Udvardi M, Katinakis P (2003) A sucrose transporter, LjSUT4, is up-regulated during *Lotus japonicus* nodule development. J Exp Bot 54:1789–1791

Gomez C, Terrier N, Torregrosa L, Vialet S, Fournier-Level A, Verries C, Souquet JM, Mazauric JP, Klein M, Cheynier V, Ageorges A (2009) Grapevine MATE-type proteins act as vacuolar H^+-dependent acylated anthocyanin transporters. Plant Physiol 150:402–415

Goodchild DJ, Bergersen FJ (1966) Electron microscopy of the infection and subsequent development of soybean nodule cells. J Bacteriol 92:204–213

Hakoyama T, Niimi K, Watanabe H, Tabata R, Matsubara J, Sato S, Nakamura Y, Tabata S, Jichun L, Matsumoto T, Tatsumi K, Nomura M, Tajima S, Ishizaka M, Yano K, Imaizumi-Anraku H, Kawaguchi M, Kouchi H, Suganuma N (2009) Host plant genome overcomes the lack of a bacterial gene for symbiotic nitrogen fixation. Nature 462:514–517

Hakoyama T, Niimi K, Yamamoto T, Isobe S, Sato S, Nakamura Y, Tabata S, Kumagai H, Umehara Y, Brossuleit K, Petersen TR, Sandal N, Stougaard J, Udvardi MK, Tamaoki M, Kawaguchi M, Kouchi H, Suganuma N (2012) The integral membrane protein SEN1 is required for symbiotic nitrogen fixation in *Lotus japonicus* nodules. Plant Cell Physiol 53: 225–236

Herridge DF, Peoples MB, Boddey RM (2008) Global inputs of biological nitrogen fixation in agricultural systems. Plant Soil 311:1–18

Jasinski M, Stukkens Y, Degand H, Purnelle B, Marchand-Brynaert J, Boutry M (2001) A plant plasma membrane ATP binding cassette-type transporter is involved in antifungal terpenoid secretion. Plant Cell 13:1095–1107

Jasinski M, Banasiak J, Radom M, Kalitkiewicz A, Figlerowicz M (2009) Full-size ABC transporters from the ABCG subfamily in *Medicago truncatula*. Mol Plant Microbe Interact 22: 921–931

Kaiser BN, Moreau S, Castelli J, Thomson R, Lambert A, Bogliolo S, Puppo A, Day DA (2003) The soybean NRAMP homologue, GmDMT1, is a symbiotic divalent metal transporter capable of ferrous iron transport. Plant J 35:295–304

Kamimoto Y, Terasaka K, Hamamoto M, Takanashi K, Fukuda S, Shitan N, Sugiyama A, Suzuki H, Shibata D, Wang B, Pollmann S, Geisler M, Yazaki K (2012) Arabidopsis ABCB21 is a facultative auxin importer/exporter regulated by cytoplasmic auxin concentration. Plant Cell Physiol 53:2090–2100

Kang J, Hwang JU, Lee M, Kim YY, Assmann SM, Martinoia E, Lee Y (2010) PDR-type ABC transporter mediates cellular uptake of the phytohormone abscisic acid. Proc Natl Acad Sci U S A 107:2355–2360

Kanno S, Yamawaki M, Ishibashi H, Kobayashi NI, Hirose A, Tanoi K, Nussaume L, Nakanishi TM (2012) Development of real-time radioisotope imaging systems for plant nutrient uptake studies. Philos Trans R Soc Lond B Biol Sci 367:1501–1508

Kosslak RM, Bookland R, Barkei J, Paaren HE, Appelbaum ER (1987) Induction of Bradyrhizobium japonicum common nod genes by isoflavones isolated from *Glycine max*. Proc Natl Acad Sci U S A 84:7428–7432

Kouchi H, Shimomura K, Hata S, Hirota A, Wu GJ, Kumagai H, Tajima S, Suganuma N, Suzuki A, Aoki T, Hayashi M, Yokoyama T, Ohyama T, Asamizu E, Kuwata C, Shibata D, Tabata S (2004) Large-scale analysis of gene expression profiles during early stages of root nodule formation in a model legume, *Lotus japonicus*. DNA Res 11:263–274

Kretzschmar T, Kohlen W, Sasse J, Borghi L, Schlegel M, Bachelier JB, Reinhardt D, Bours R, Bouwmeester HJ, Martinoia E (2012) A petunia ABC protein controls strigolactone-dependent symbiotic signalling and branching. Nature 483:341–344

Krusell L, Krause K, Ott T, Desbrosses G, Kramer U, Sato S, Nakamura Y, Tabata S, James EK, Sandal N, Stougaard J, Kawaguchi M, Miyamoto A, Suganuma N, Udvardi MK (2005) The sulfate transporter SST1 is crucial for symbiotic nitrogen fixation in *Lotus japonicus* root nodules. Plant Cell 17:1625–1636

Lerouge P, Roche P, Faucher C, Maillet F, Truchet G, Prome JC, Denarie J (1990) Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamine oligosaccharide signal. Nature 344:781–784

LeVier K, Day DA, Guerinot ML (1996) Iron uptake by symbiosomes from soybean root nodules. Plant Physiol 111:893–900

Lewis DR, Miller ND, Splitt BL, Wu G, Spalding EP (2007) Separating the roles of acropetal and basipetal auxin transport on gravitropism with mutations in two *Arabidopsis multidrug resistance-like* ABC transporter genes. Plant Cell 19:1838–1850

Libault M, Farmer A, Joshi T, Takahashi K, Langley RJ, Franklin LD, He J, Xu D, May G, Stacey G (2010) An integrated transcriptome atlas of the crop model *Glycine max*, and its use in comparative analyses in plants. Plant J 63:86–99

Luo B, Xue XY, Hu WL, Wang LJ, Chen XY (2007) An ABC transporter gene of *Arabidopsis thaliana*, AtWBC11, is involved in cuticle development and prevention of organ fusion. Plant Cell Physiol 48:1790–1802

Marinova K, Pourcel L, Weder B, Schwarz M, Barron D, Routaboul JM, Debeaujon I, Klein M (2007) The Arabidopsis MATE transporter TT12 acts as a vacuolar flavonoid/H^+-antiporter active in proanthocyanidin-accumulating cells of the seed coat. Plant Cell 19:2023–2038

Mathesius U (2008) Auxin: at the root of nodule development? Funct Plant Biol 35:651–668

McKay IA, Djordjevic MA (1993) Production and excretion of Nod metabolites by *Rhizobium leguminosarum* bv. trifolii are disrupted by the same environmental factors that reduce nodulation in the field. Appl Environ Microbiol 59:3385–3392

Moreau S, Thomson RM, Kaiser BN, Trevaskis B, Guerinot ML, Udvardi MK, Puppo A, Day DA (2002) GmZIP1 encodes a symbiosis-specific zinc transporter in soybean. J Biol Chem 277: 4738–4746

Mulligan JT, Long SR (1985) Induction of *Rhizobium meliloti* nodC expression by plant exudate requires nodD. Proc Natl Acad Sci U S A 82:6609–6613

Murray JD (2011) Invasion by invitation: rhizobial infection in legumes. Mol Plant Microbe Interact 24:631–639

Oldroyd GE, Murray JD, Poole PS, Downie JA (2011) The rules of engagement in the legume-rhizobial symbiosis. Annu Rev Genet 45:119–144

Oldroyd GE (2013) Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants. Nat Rev Microbiol 11:252–263

Otani M, Shitan N, Sakai K, Martinoia E, Sato F, Yazaki K (2005) Characterization of vacuolar transport of the endogenous alkaloid berberine in *Coptis japonica*. Plant Physiol 138:1939–1946

Pacios-Bras C, Schlaman HRM, Boot K, Admiraal P, Langerak JM, Stougaard J, Spaink HP (2003) Auxin distribution in *Lotus japonicus* during root nodule development. Plant Mol Biol 52:1169–1180

Panikashvili D, Savaldi-Goldstein S, Mandel T, Yifhar T, Franke RB, Hofer R, Schreiber L, Chory J, Aharoni A (2007) The Arabidopsis *DESPERADO/AtWBC11* transporter is required for cutin and wax secretion. Plant Physiol 145:1345–1360

Panter S, Thomson R, de Bruxelles G, Laver D, Trevaskis B, Udvardi M (2000) Identification with proteomics of novel proteins associated with the peribacteroid membrane of soybean root nodules. Mol Plant Microbe Interact 13:325–333

Peters NK, Frost JW, Long SR (1986) A plant flavone, luteolin, induces expression of Rhizobium meliloti nodulation genes. Science 233:977–980

Pueppke SG, Bolanos-Vasquez MC, Werner D, Bec-Ferte MP, Prome JC, Krishnan HB (1998) Release of flavonoids by the soybean cultivars McCall and peking and their perception as signals by the nitrogen-fixing symbiont *Sinorhizobium fredii*. Plant Physiol 117:599–606

Redmond JW, Batley M, Djordjevic MA, Innes RW, Kuempel PL, Rolfe BG (1986) Flavones induce expression of nodulation genes in rhizobium. Nature 323:632–635

Reinders A, Sivitz AB, Starker CG, Gantt JS, Ward JM (2008) Functional analysis of LjSUT4, a vacuolar sucrose transporter from *Lotus japonicus*. Plant Mol Biol 68:289–299

Rossen L, Shearman CA, Johnston AW, Downie JA (1985) The nodD gene of Rhizobium leguminosarum is autoregulatory and in the presence of plant exudate induces the nodA, B, C genes. EMBO J 4:3369–3373

Roth LE, Stacey G (1989) Bacterium release into host cells of nitrogen-fixing soybean nodules: the symbiosome membrane comes from three sources. Eur J Cell Biol 49:13–23

Saalbach G, Erik P, Wienkoop S (2002) Characterisation by proteomics of peribacteroid space and peribacteroid membrane preparations from pea (*Pisum sativum*) symbiosomes. Proteomics 2: 325–337

Saier MH Jr, Tam R, Reizer A, Reizer J (1994) Two novel families of bacterial membrane proteins concerned with nodulation, cell division and transport. Mol Microbiol 11:841–847

Sanchez-Fernandez R, Davies TGE, Coleman JOD, Rea PA (2001) The *Arabidopsis thaliana* ABC protein superfamily, a complete inventory. J Biol Chem 276:30231–30244

Santelia D, Vincenzetti V, Azzarello E, Bovet L, Fukao Y, Duchtig P, Mancuso S, Martinoia E, Geisler M (2005) MDR-like ABC transporter AtPGP4 is involved in auxin-mediated lateral root and root hair development. FEBS Lett 579:5399–5406

Sato S, Nakamura Y, Kaneko T, Asamizu E, Kato T, Nakao M, Sasamoto S, Watanabe A, Ono A, Kawashima K, Fujishiro T, Katoh M, Kohara M, Kishida Y, Minami C, Nakayama S, Nakazaki N, Shimizu Y, Shinpo S, Takahashi C, Wada T, Yamada M, Ohmido N, Hayashi M, Fukui K, Baba T, Nakamichi T, Mori H, Tabata S (2008) Genome structure of the Legume, *Lotus japonicus*. DNA Res 15:227–239

Schmidt J, Wingender R, John M, Wieneke U, Schell J (1988) *Rhizobium meliloti* nodA and nodB genes are involved in generating compounds that stimulate mitosis of plant cells. Proc Natl Acad Sci U S A 85:8578–8582

Schmutz J, Cannon SB, Schlueter J, Ma J, Mitros T, Nelson W, Hyten DL, Song Q, Thelen JJ, Cheng J, Xu D, Hellsten U, May GD, Yu Y, Sakurai T, Umezawa T, Bhattacharyya MK, Sandhu D, Valliyodan B, Lindquist E, Peto M, Grant D, Shu S, Goodstein D, Barry K, Futrell-Griggs M, Abernathy B, Du J, Tian Z, Zhu L, Gill N, Joshi T, Libault M, Sethuraman A, Zhang XC, Shinozaki K, Nguyen HT, Wing RA, Cregan P, Specht J, Grimwood J, Rokhsar D, Stacey G, Shoemaker RC, Jackson SA (2010) Genome sequence of the palaeopolyploid soybean. Nature 463:178–183

Shitan N, Bazin I, Dan K, Obata K, Kigawa K, Ueda K, Sato F, Forestier C, Yazaki K (2003) Involvement of CjMDR1, a plant multidrug-resistance-type ATP-binding cassette protein, in alkaloid transport in *Coptis japonica*. Proc Natl Acad Sci U S A 100:751–756

Simon-Rosin U, Wood C, Udvardi MK (2003) Molecular and cellular characterisation of LjAMT2;1, an ammonium transporter from the model legume *Lotus japonicus*. Plant Mol Biol 51:99–108

Spaink HP, Wijfjes AH, Lugtenberg BJ (1995) Rhizobium NodI and NodJ proteins play a role in the efficiency of secretion of lipochitin oligosaccharides. J Bacteriol 177:6276–6281

Sprent JI (2007) Evolving ideas of legume evolution and diversity: a taxonomic perspective on the occurrence of nodulation. New Phytol 174:11–25

Stukkens Y, Bultreys A, Grec S, Trombik T, Vanham D, Boutry M (2005) NpPDR1, a pleiotropic drug resistance-type ATP-binding cassette transporter from *Nicotiana plumbaginifolia*, plays a major role in plant pathogen defense. Plant Physiol 139:341–352

Suganuma N, Nakamura Y, Yamamoto M, Ohta T, Koiwa H, Akao S, Kawaguchi M (2003) The *Lotus japonicus Sen1* gene controls rhizobial differentiation into nitrogen-fixing bacteroids in nodules. Mol Genet Genom 269:312–320

Sugiyama A, Shitan N, Sato S, Nakamura Y, Tabata S, Yazaki K (2006) Genome-wide analysis of ATP-binding cassette (ABC) proteins in a model legume plant, *Lotus japonicus*: comparison with *Arabidopsis* ABC protein family. DNA Res 13:205–228

Sugiyama A, Shitan N, Yazaki K (2007) Involvement of a soybean ATP-binding cassette - type transporter in the secretion of genistein, a signal flavonoid in legume-*Rhizobium* symbiosis. Plant Physiol 144:2000–2008

Suzaki T, Yano K, Ito M, Umehara Y, Suganuma N, Kawaguchi M (2012) Positive and negative regulation of cortical cell division during root nodule development in *Lotus japonicus* is accompanied by auxin response. Development 139:3997–4006

Suzuki H, Takahashi S, Watanabe R, Fukushima Y, Fujita N, Noguchi A, Yokoyama R, Nishitani K, Nishino T, Nakayama T (2006) An isoflavone conjugate-hydrolyzing β-glucosidase from the roots of soybean (*Glycine max*) seedlings. J Biol Chem 281:30251–30259

Szczyglowski K, Kapranov P, Hamburger D, de Bruijn FJ (1998) The *Lotus japonicus* LjNOD70 nodulin gene encodes a protein with similarities to transporters. Plant Mol Biol 37:651–661

Takanashi K, Sugiyama A, Yazaki K (2011) Involvement of auxin distribution in root nodule development of *Lotus japonicus*. Planta 234:73–81

Takanashi K, Sugiyama A, Sato S, Tabata S, Yazaki K (2012a) LjABCB1, an ATP-binding cassette protein specifically induced in uninfected cells of *Lotus japonicus* nodules. J Plant Physiol 169:322–326

Takanashi K, Takahashi H, Sakurai N, Sugiyama A, Suzuki H, Shibata D, Nakazono M, Yazaki K (2012b) Tissue-specific transcriptome analysis in nodules of *Lotus japonicus*. Mol Plant Microbe Interact 25:869–876

Takanashi K, Yokosho K, Saeki K, Sugiyama A, Sato S, Tabata S, Ma JF, Yazaki K (2013) LjMATE1: a citrate transporter responsible for iron supply to the nodule infection zone of *Lotus japonicus*. Plant Cell Physiol 54:585–594

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739

Terasaka K, Blakeslee JJ, Titapiwatanakun B, Peer WA, Bandyopadhyay A, Makam SN, Lee OR, Richards EL, Murphy AS, Sato F, Yazaki K (2005) PGP4, an ATP binding cassette p-glycoprotein, catalyzes auxin transport in *Arabidopsis thaliana* roots. Plant Cell 17: 2922–2939

Tsuyama T, Kawai R, Shitan N, Matoh T, Sugiyama J, Yoshinaga A, Takabe K, Fujita M, Yazaki K (2013) Proton-dependent coniferin transport, a common major transport event in differentiating xylem tissue of woody plants. Plant Physiol 162:918–926

Tyerman SD, Whitehead LF, Day DA (1995) A channel-like transporter for NH_4^+ on the symbiotic interface of N_2-fixing plants. Nature 378:629–632

Udvardi MK, Day DA (1989) Electrogenic ATPase activity on the peribacteroid membrane of soybean (*Glycine max* L.) root nodules. Plant Physiol 90:982–987

Udvardi M, Poole PS (2013) Transport and metabolism in legume-rhizobia symbioses. Annu Rev Plant Biol 64:781–805

Udvardi MK, Price GD, Gresshoff PM, Day DA (1988) A dicarboxylate transporter on the peribacteroid membrane of soybean nodules. FEBS Lett 231:36–40

Udvardi MK, Yang LJO, Young S, Day DA (1990) Sugar and amino-acid-transport across symbiotic membranes from soybean nodules. Mol Plant Microbe Interact 3:334–340

Udvardi MK, Lister DL, Day DA (1991) ATPase activity and anion transport across the peribacteroid membrane of isolated soybean symbiosomes. Arch Microbiol 156:362–366

Ukitsu H, Kuromori T, Toyooka K, Goto Y, Matsuoka K, Sakuradani E, Shimizu S, Kamiya A, Imura Y, Yuguchi M, Wada T, Hirayama T, Shinozaki K (2007) Cytological and biochemical analysis of *COF1*, an *Arabidopsis* mutant of an ABC transporter gene. Plant Cell Physiol 48: 1524–1533

Vasse J, de Billy F, Camut S, Truchet G (1990) Correlation between ultrastructural differentiation of bacteroids and nitrogen fixation in alfalfa nodules. J Bacteriol 172:4295–4306

Verrier PJ, Bird D, Burla B, Dassa E, Forestier C, Geisler M, Klein M, Kolukisaoglu U, Lee Y, Martinoia E, Murphy A, Rea PA, Samuels L, Schulz B, Spalding EJ, Yazaki K, Theodoulou FL (2008) Plant ABC proteins - a unified nomenclature and updated inventory. Trends Plant Sci 13:151–159

Vincill ED, Szczyglowski K, Roberts DM (2005) GmN70 and LjN70. Anion transporters of the symbiosome membrane of nodules with a transport preference for nitrate. Plant Physiol 137: 1435–1444

Wienkoop S, Saalbach G (2003) Proteome analysis. Novel proteins identified at the peribacteroid membrane from *Lotus japonicus* root nodules. Plant Physiol 131:1080–1090

Yoshimura Y, Enomoto H, Moriyama T, Kawamura Y, Setou M, Zaima N (2012) Visualization of anthocyanin species in rabbiteye blueberry *Vaccinium ashei* by matrix-assisted laser desorption/ionization imaging mass spectrometry. Anal Bioanal Chem 403:1885–1895

Young ND, Debelle F, Oldroyd GE, Geurts R, Cannon SB, Udvardi MK, Benedito VA, Mayer KF, Gouzy J, Schoof H, Van de Peer Y, Proost S, Cook DR, Meyers BC, Spannagl M, Cheung F, De Mita S, Krishnakumar V, Gundlach H, Zhou S, Mudge J, Bharti AK, Murray JD, Naoumkina MA, Rosen B, Silverstein KA, Tang H, Rombauts S, Zhao PX, Zhou P, Barbe V, Bardou P, Bechner M, Bellec A, Berger A, Berges H, Bidwell S, Bisseling T, Choisne N, Couloux A, Denny R, Deshpande S, Dai X, Doyle JJ, Dudez AM, Farmer AD, Fouteau S, Franken C, Gibelin C, Gish J, Goldstein S, Gonzalez AJ, Green PJ, Hallab A, Hartog M, Hua A, Humphray SJ, Jeong DH, Jing Y, Jocker A, Kenton SM, Kim DJ, Klee K, Lai H, Lang C, Lin S, Macmil SL, Magdelenat G, Matthews L, McCorrison J, Monaghan EL, Mun JH, Najar FZ, Nicholson C, Noirot C, O'Bleness M, Paule CR, Poulain J, Prion F, Qin B, Qu C, Retzel EF, Riddle C, Sallet E, Samain S, Samson N, Sanders I, Saurat O, Scarpelli C, Schiex T, Segurens B, Severin AJ, Sherrier DJ, Shi R, Sims S, Singer SR, Sinharoy S, Sterck L, Viollet A, Wang BB, Wang K, Wang M, Wang X, Warfsmann J, Weissenbach J, White DD, White JD, Wiley GB, Wincker P, Xing Y, Yang L, Yao Z, Ying F, Zhai J, Zhou L, Zuber A, Denarie J, Dixon RA, May GD, Schwartz DC, Rogers J, Quetier F, Town CD, Roe BA (2011) The *Medicago* genome provides insight into the evolution of rhizobial symbioses. Nature 480: 520–524

Yu F, De Luca V (2013) ATP-binding cassette transporter controls leaf surface secretion of anticancer drug components in *Catharanthus roseus*. Proc Natl Acad Sci U S A 110: 15830–15835

Zhao J, Dixon RA (2009) MATE transporters facilitate vacuolar uptake of epicatechin 3'-*O*-glucoside for proanthocyanidin biosynthesis in *Medicago truncatula* and *Arabidopsis*. Plant Cell 21:2323–2340

Monocot ABC Transporters

YanXia Xu and YanHua Qi

Abstract The ABC transporter family is large and widespread in all organisms. In plants, this family is usually highly expanded with more than 100 ABC transporters that can be divided into eight subfamilies (ABCA–ABCG and ABCI). Significant progress has been made in the structural and functional characterization of ABC genes in the last 20 years, not only in dicots but also in monocots.

In this review, we focused on the research of ABC transporter in monocots. ABCB genes of maize and sorghum play an important role in stalk growth through modulating polar auxin transport. ZmABCC3 and ZmABCC4 function in anthocyanin transport. Additionally, ZmABCC4 and its homologous genes in soybean and rice are involved in phytic acid accumulation. Wheat ABCG transporter LR34 was associated with robust and durable resistance to the diseases, whereas barley ABCG transporter, HvABCG31, plays an essential role in leaf water conservation associated to the cutin formation. Rice ABCG15 plays an important role in the transport of anther cuticle and sporopollenin precursors whereas ABCG5 is involved in controlling shoot branching. OsSTAR1 and OsSTAR2 are members of ABCI subfamily with implications in aluminum toxicity response. Compared to this, little is know about the members of ABCA, ABCD, ABCE, ABCF, and ABCI subfamilies in monocots.

Eukaryotic ABC transporters consist of two hydrophobic transmembrane domains (TMDs) contributing to the substrate translocation pathway and two nucleotide-binding domains (NBDs) involved in ATP binding. TMDs contain several membrane-spanning α-helices and NBDs contain several highly conserved motifs: Walker A, Walker B, ABC signature, H loop, and the Q loop. Prokaryotic ABC importers have a fifth domain: the substrate-binding protein (SBP) responsible for the substrate specificity of ABC importers (Higgins and Linton, Nat Struct Mol Biol 11:918–926, 2004).

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On the basis of sequence similarity, protein size (full, half or quarter molecule), orientation (forward or reverse pattern), and the presence or absence of idiotypic transmembrane and/or linker domains, the plant ABC proteins can be divided into 8 subfamilies (ABCA–ABCG and ABCI) according to Verrier et al. (Trends Plant Sci 13:151–159, 2008) who introduced the systematical nomenclature for plants ABC proteins.

1 ABCA Subfamily

Several members of ABCA subfamily in mammalian are associated with severe health disorders, such as Tangier disease T1, familial high-density lipoprotein deficiency, Stargardt disease-1, age-related macular degeneration, and retinitis pigmentosa (Vasiliou et al. 2009). However, there is only little information on plant members of this subfamily.

Re-annotation of the Arabidopsis and rice reveals a difference between the two species. The ABCA subfamily of Arabidopsis is composed of 12 members of which 11 are half-molecular ABCA genes (alias ATH) and one is a full-length ABCA gene (alias AOH), while the rice ABCA subfamily is constituted by six half-size ABCA genes (ATH), lacking full-length ABCA gene (Jasinski et al. 2003; Garcia et al. 2004). Blast analysis of five plant species genomes (total 35 plants in Arabidopsis, Sorghum bicolor, *Zea mays*, *Oryza sativa* and *Vitis vinifera*) displayed that full-length ABCA genes (AOHs) existed in all embryophytes except monocot grasses, not only rice but also sorghum and maize (Pang et al. 2013). This may indicate a gene lose event when the monocot/dicot split, considering that AOH orthologues have been found in *Physcomitrella patens,* a moss that be the representative of the basal land plants (Rensing et al. 2008). ATH is the only half-size plant ABC transporter category not represented in yeast. However, although the studies of ABCAs in animals are intensive, there is not much known in plants. The only thing we know about plant ABCAs is that NaCl can reduce the expression of AtATH14 and AtATH15 based on transcriptome analyses of root transporters (Maathuis et al. 2003).

2 ABCB Subfamily

The subfamily B of plant ABC transporter consists of four subgroups of proteins, full-molecule multidrug-resistance/p-glycoprotein (MDR/PGP) proteins, half-molecule transporter associated with antigen processing (TAP) proteins, half-molecule ABC transporter of the mitochondria (ATM) proteins, and half-molecule lipid A-like exporter putative (LLP) proteins (Verrier et al. 2008). ABCBs are the

second largest subfamily of ABC proteins in plants and they have been verified to play a crucial role in many key developmental events in plants (Rea 2007; Kang et al. 2011). Plant ABCB genes were initially described to regulate hypocotyl cell elongation under dim light in 1998 (Sidler et al. 1998) and have since been widely studied in plants (see chapters "Monocot ABC transporters", "Structure-function of plant ABC-transporters", "It takes more than two to tango: Regulation of plant ABC transporters", "Evolution of transport directionality in ABCBs", "Trafficking of ABCB-type auxin transporters", "Function of ABCBs in light signaling").

AtABCB1 and AtABCB19 are the best-studied examples of the ABCB transporters in *Arabidopsis*. Data obtained in experiments with *atpgp19* mutants and overexpression lines provided first evidence that AtABCB19/AtPGP19/MDR1 is a putative auxin efflux carrier (Noh et al. 2001). First, AtPGP19 was shown to be induced by IAA (Noh et al. 2001). Second, loss-of-function mutants for *AtPGP19* gene displayed an auxin-related phenotypes: epinastic cotyledons, curled leaves, dwarf shoots, and reduced apical dominance. The phenotypes could be rescued by molecular complementation of the *atpgp19* mutants (Noh et al. 2001). Third, AtPGP19 could bind tightly and specifically to the auxin transport inhibitor 1-naphthylphthalamic acid (NPA), and the effect of the *AtPGP19* mutations on IAA transport could be mimicked in wild type by NPA treatment (Noh et al. 2001). Last, the root hairs of *AtPGP19* overexpression lines was shorter than that of wild type when taking advantage of the root hair cell system that could be used to study the activity of auxin transporter (Cho et al. 2007) and AtABCB19 was localized to the plasma membrane and expressed in special tissues such as the endodermis and the pericycle of roots (Lewis et al. 2007; Wu et al. 2007). The most direct biochemical evidences for auxin efflux function of AtPGP19 has been obtained by Bouchard et al. (2006), who characterized AtABCB19 in Hela cells and plant protoplasts. The two systems proved that AtABCB19 functions as an ATP-driven auxin efflux transporter.

For now, in the Arabidopsis ABCB family, only nine ABCB genes (AtABCB1, AtABCB4, AtABCB14, AtABCB19, AtABCB21, AtABCB23, AtABCB24, AtABCB25, and AtABCB27) have been functionally characterized. Of them, two are auxin efflux transporters (mentioned above): AtABCB19 and AtABCB1 (Sidler et al. 1998; Noh et al. 2001; Geisler et al. 2003, 2005; Wang et al. 2013a); two are facultative auxin im/exporters: AtABCB4 and AtABCB21 (Terasaka et al. 2005; Kamimoto et al. 2012; Kubeš et al. 2012); one is malate uptake transporter: AtABCB14 (Lee et al. 2008) and three are involved in iron homeostasis: AtABCB23, AtABCB24, AtABCB25 (AtABCB25 is recently reported to be also involved in heavy metal resistance and molybdenum cofactor biosynthesis (Kim et al. 2006; Chen et al. 2007; Bernard et al. 2009; Teschner et al. 2010)) and one is important for Al sequestration: AtABCB27 (Larsen et al. 2007).

Rice, maize, and sorghum are three of the most important cereal crops, providing a food staple to a large human population and livestock in the world. Along with the completion of sequencing of the entire genomes of the above species, people have gained a primary understanding of ABCBs' classification, basic structure, typical function, evolution track, and expression profiles in these species (Jasinski

et al. 2003; Shen et al. 2010; Pang et al. 2013). Even so, little is known about their real biological function.

Regarding the function of ABCBs in monocotyledons, there is a small number of evidence that they also involve in the transport of IAA, aluminum toxicity responses, and calcium homeostasis. Barley *HvMDR2* was first cloned and preliminarily characterized (Davies et al. 1997). Davies et al. (1997) reported that *HvMDR2* was expressed at low levels in both barley roots and leaves, but they didn't report the detailed function about *HvMDR2*. Later, wheat *TaMDR1* was reported to plausible function in Al toxicity responses and calcium homeostasis, because *TaMDR1* was induced in wheat root apices by aluminum and the Al concentration required for the induction was lower in the Al-sensitive cultivar than in the Al-tolerant cultivar; and *TaMDR1* was also induced by the inhibitors of calcium channels and the reduced content of calcium ion (Sasaki et al. 2002). Further detailed evidence for ABCB function in monocotyledon was provided by Multani et al. (2003) demonstrating that the defect of MDR genes in maize and sorghum resulted in impaired auxin transport, thereby leading to a dwarf phenotype as shown by the *brachytic2* and *dwarfing3* mutants (Multani et al. 2003; Knoller et al. 2010) (Fig. 1). Additionally, recent evidence has also shown that ABCB/PGP-like gene products play a key role in regulation of the direction of auxin transport after blue light perception by phot1 in maize (Matsuda et al. 2011). Protein–protein interactions and phosphorylations are key events in that respect, which have become the field of increasing research interest in Arabidopsis in the recent years (Geisler et al. 2003, 2004; Bouchard et al. 2006; Bailly et al. 2008; Wu et al. 2010; Christie et al. 2011; Henrichs et al. 2012; Wang et al. 2013a). Therefore it is reasonbale to assume that similar mechanisms will be reported also in monocots in the future.

3 ABCC Subfamily

The ABCC subfamily is also called multidrug resistance-associated protein (MRPs) subfamily and is roughly similar to MDRs in protein structure. They differ in the N-terminal region where ABCCs/MRPs have an extremely hydrophobic N-terminal extension (NTE or TMD0) with unknown function, but MDRs do not (Klein et al. 2006). The subfamily C of Arabidopsis, rice and maize contains 15 (denoted AtABCC1-15), 17 (denoted OsABCC1-17) and 13 (denoted ZmABCC1-13) members, respectively, all of which appear to be full-size ABC proteins (Verrier et al. 2008; Pang et al. 2013).

The human HsABCC1 protein was reported to catalyze the transport of glutathione (GSH) conjugates (GS-conjugates). In analogy to the research on human ABCC, many early studies of plant ABCCs focused on the transport of GS-conjugates (*N*-ethylmaleimide-GS and S-(2,4-dinitrophenyl)-G) and the glutathionated chloroacetanilide herbicide (metolachlor-GS) (Martinoia et al. 1993; Li et al. 1995; see Chap. "ABC transporters and heavy metals"). As

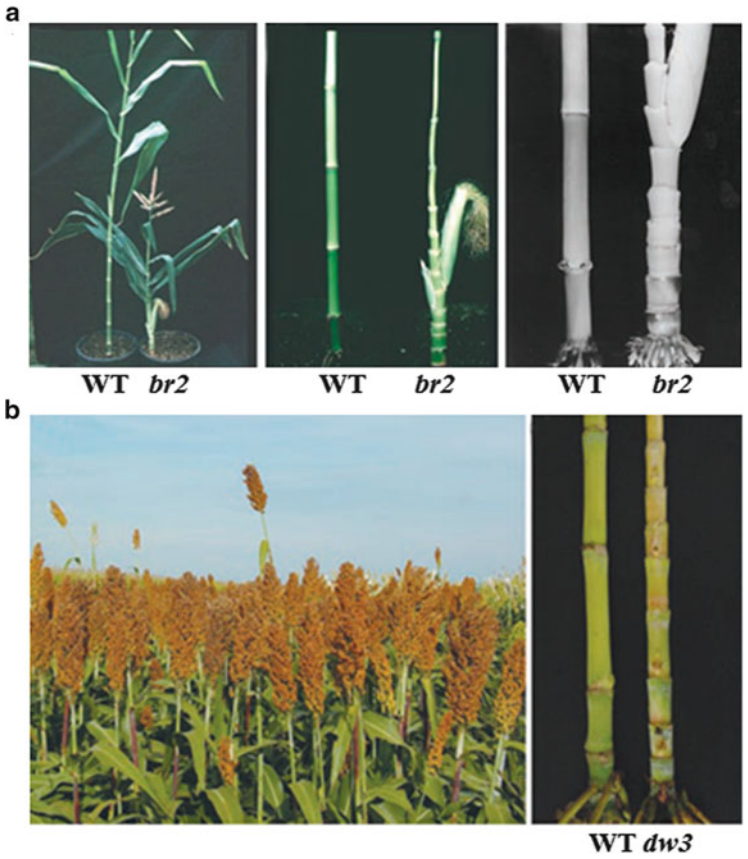

Fig. 1 *brachytic2* (*br2*) and *dwarfing* 3 (*dw3*) mutants with a defect in ABCB genes in maize (**a**) and sorghum (**b**) display a dwarf phenotype resulted from impaired auxin transport. Images were taken from Multani et al. (2003)

early as the report of functional characterizing maize bronze-2 gene, published in 1995, researchers suggested a possible connection between ABCC genes and the vacuolar transport of anthocyanin (Marrs et al. 1995). The *bronze-2* (*bz2*) mutant was defective in the accumulation of the anthocyanin in its vacuolar, resulting in a bronze color in tissues. Given that previous studies indicated that GS-conjugates were good substrate for ABCC in vitro, the involvement of ABCC in the vacuolar transport of anthocyanin in vivo was presumed. This speculation was confirmed by the research on AtMRP1 and AtMRP2 (Lu et al. 1997, 1998).

ZmMRP3 and ZmMRP4 were the best identified genes of ABC family in monocot plants and also the first for whom the in vivo transport of a specific endogenous substrate was described. A mutant line for ZmMRP3 exhibited a distinct pigmentation phenotype in the adult plant, consistent with the mislocalization of pigment and reduced anthocyanin content. Moreover,

ZmMRP3 was localized in the tonoplast at where anthocyanin transport occurs and its promoter is regulated by the maize anthocyanin transcription factors, all of these suggested that ZmMRP3 function on anthocyanin transport. ZmMRP4 is also involved in the same function (Goodman et al. 2004).

Later, several mutations have been isolated in this gene ZmMRP4 resulting in a reduction of phytic acid content and an equivalent increasing of available phosphate content, more importantly, no any significant reduction in seed dry weight (Raboy 2007; Shi et al. 2007; Badone et al. 2010; Cerino Badone et al. 2012). ZmMRP4's involving in phytic acid accumulation has a profound significance. Cereal grains, like sorghum, maize, rice, and wheat, contain large quantities of phytic acid (inositol hexaphosphate), which provides essential myo-inositol and phosphorus required during seed germination and seedling growth. But phytic acid cannot be digested efficiently by non-ruminants, effecting agriculture. On the other hand, phytic acid chelates divalent cations, such as Fe^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+}, and Ca^{2+}, having an impact on human health. ZmMRP4's involvement in phytic acid accumulation provides a profitable and environmentally friendly approach to engineering and breeding low-phytate varieties (Raboy 2007).

In addition, suppression of the homologous genes in soybean and rice led to a similar alteration in the content of phytic acid and free phosphate (Shi et al. 2007; Xu et al. 2009), suggesting a conserved function for this gene in plants.

4 ABCD Subfamily

The yeast and animal ABCD subfamily (also known as peroxisomal membrane protein (PMP)) contain forward-oriented, half-molecule ABC transporters that catalyze the transport of long-chain acylcoenzyme A substrates (Wanders et al. 2007; see also chapter "Plant peroxisomal ABC transporters: flexible and unusual"). In plants, the ABCD subfamily consists of half-size and full-size ABC transporters. The Arabidopsis genome encodes two members of ABCDs, comparing to three and four members in the rice and maize genome, respectively (Pang et al. 2013). Of them, one member of Arabidopsis, two member of rice, and two member of maize are full-size, a feature that is exceptional in eukaryotes. Plant ABCD transporters were supposed to function on transport of the long-chain fatty acids across the peroxisomal membrane (Hettema and Tabak 2000).

AtABCD1 is the best-characterized ABCD transporter in plants. Several lines of evidence indicated that, AtABCD1 is responsible for importing a lot of substrates, such as fatty acyl-CoA, precursors of auxin and jasmonic acid and acetate (Russell et al. 2000; Zolman et al. 2001; Footitt et al. 2002; Hayashi et al. 2002; Theodoulou et al. 2005; Hooks et al. 2007; Dietrich et al. 2009; Nyathi et al. 2010). However, functions of these transporters in monocots are not yet reported, but some data from researchers are useful to speculate on their putative function. For example, OsABCD1 is highly homologous to cyanobacterial ABC transporters, which were involved in the export of toxic cyclic peptide-polyketides (Tillett et al. 2000).

Phylogenetic analysis between five species (Arabidopsis, rice, sorghum, maize, and grape) showed that monocots have evolved two exclusive branches of genes and these genes are highly conservative across monocot species, suggesting that ABCD genes in these branches have similar function across monocot species that is different from Arabidopsis. ZmABCD4 and ZmABCD1 are prominently expressed in growing points, while ZmABCD2 was distributed to leaf tissue. ZmABCD3 exhibits primary expression at later seedling stage, similar to that in Arabidopsis (Pang et al. 2013).

5 ABCE/ABCF Subfamily

With 3/5 members in Arabidopsis, 2/5 members in rice and 2/7 members in maize (Pang et al. 2013), the plant ABCE and ABCF proteins are classified into soluble ABC proteins and do not adhere to any membrane (Karcher et al. 2005). Plant ABCE subfamily (alias rnase-L inhibitor (RLI) subfamily) characterized by a ferredoxin iron–sulphur centre in the N-terminal ABC domain has been implicated to play a key role in the transcription events and the posttranslational events (Bisbal et al. 2000; Zimmerman et al. 2002; Braz et al. 2004; Chen et al. 2006; Bessard et al. 2009). And plant ABCF subfamily is also known as regulation of gene expression (REG) subfamily that has been implicated to regulate the gene expression (Winans et al. 1988; Marton et al. 1997; Liu et al. 2001). However, nothing is reported on the function of members of both subfamilies in monocots until now.

6 ABCG Subfamily

The ABCG subfamily constitutes the largest subfamily of ABCs in plants (43 members in Arabidopsis, 51 members in rice and 54 members in maize) and it contains the reverse-oriented full-sized pleiotropic drug resistance homologs (PDRs) and half-sized white–brown complex homolog proteins (WBCs) (Verrier et al. 2008; Pang et al. 2013).

Since the first functional characterization of an ABCG gene, the *Spirodela polyrrhiza* TUR2 gene, a large number of studies on plant ABCGs have been conducted (van den Brule et al. 2002). Expression and function of plant ABCG gene is predominantly studied in Arabidopsis and they function variously, such as in the export and translocation of sporopollenin precursors (AtABCG26) (Quilichini et al. 2010; Choi et al. 2011; Dou et al. 2011; see chapter "Exine Export In Pollen" for details); lipid export (AtABCG11, AtABCG12, AtABCG13, AtABCG32) (Pighin et al. 2004; Bird et al. 2007; Panikashvili et al. 2007, 2010, 2011; Bessire et al. 2011); ABA responses (AtABCG25, AtABCG40 and AtABCG22) (Kang et al. 2010; Kuromori et al. 2010, 2011); heavy metal resistance and pathogen defense (AtABCG36) (Kobae et al. 2006; Kim et al. 2007); plant

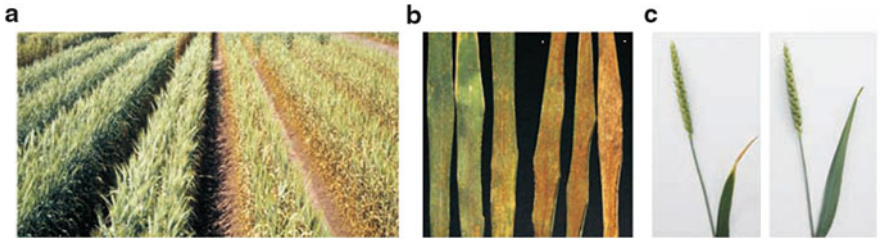

Fig. 2 A wheat ABCG transporter, leaf rust resistance34 (LR34), is involved in resistance to leaf rust. (**a**) Lr34 confers a partial slow-rusting resistance. *Left*: the resistant line Jupateco R; *right*: the susceptible near-isogenic line Jupateco S. (**b**) Flag leaves infected by leaf rust. *Left*: Jupateco R (three leaves); *right*: Jupateco S (three leaves). (**c**) Lr34 is associated with leaf tip necrosis. *Left*: the resistant near-isogenic line Arina Lr34; *right*: –Lr34 Swiss winter wheat cultivar Arina. Images were taken from Krattinger et al. (2009)

kanamycin resistance (AtABCG19) (Mentewab and Stewart 2005); and paraquat tolerance (AtABCG39) (Xi et al. 2012).

Studies in the dicotyledonous models, Arabidopsis and tobacco, were usually applied to homologous monocotyledonous agricultural crops, because these precious studies are useful for future breeding for high-yielding agricultural crops. In the rice genome, the expression of OsPDR9 and OsPDR20 was prominently induced by jasmonic acid and salicylic acid, respectively, suggesting a role in pathogen defense, whereas OsPDR3 and OsPDR6's induction by abscisic acid indicated a role in water deficit responses (Moons 2008). In addition, OsABCG43 was Cd inducible and OsPDR9 was also markedly induced by heavy metals, hypoxic stress and redox perturbations, indicating a role in abiotic stress responses (Moons 2003; Oda et al. 2011).

Functional characterizations of ABCG genes in monocots were also reported. A wheat ABCG transporter, leaf rust resistance34 (LR34), was associated with robust and durable resistance to the diseases of wheat: leaf rust (Fig. 2), stripe rust, and powdery mildew (Krattinger et al. 2009). The naturally occurring wild barley mutant *eibi1* is one of the most drought-sensitive mutants in the world that suffered from a severe level of water loss and defective cuticle and this gene for this mutation was mapped to be a member of ABCG subfamily, HvABCG31, suggesting that HvABCG31 play an important role in leaf water conservation associated to the cutin formation (Chen et al. 2004, 2009a, b, 2011).

For WBCs, a very recent report indicated that the rice ABCG15 may play an essential role in the transport of rice anther cuticle and sporopollenin precursors (Qin et al. 2013). Cereals such as rice and wheat are different from Arabidopsis in pollen structure. For example, tapetum cells have orbicules and an orbicular wall and pollen exine has free space between the tectum and nexine that are absent in Arabidopsis. Thus, mutation of genes result in the defective phenotypes in these sites will make it better and unique to character the monocot. Mutation in ABCG15 led to a phenotype of small and white anthers lacking mature pollen, lipidic cuticle, orbicules, and pollen exine and the number of wax components and aliphatic cutin

monomers in anther cuticle was significantly reduced, indicating a role in postmeiotic anther and pollen development (Qin et al. 2013). Additionally, OsABCG5 was suggested to control shoot branching by promoting the outgrowth of lateral shoots, by the analysis of *rice reduced culm number 1 (rcn1)* mutants that displayed a phenotype of prominently reduced culm number, even no any tiller after growth for 50 days (Yasuno et al. 2009).

7 ABCI Subfamily

The ABCI subfamily is composed of five subfamilies: (1) iron–sulphur center biogenesis proteins (ISBs), which have been demonstrated to be widely involved in Fe–S biosynthesis processes, metabolic reactions, signaling sensing, electron transfer, tissue senescence, regulation of gene expression, and embryo development (Kou et al. 2012; Pang et al. 2013). (2) Cytochrome c maturation proteins (CCMs), which have been reported to be responsible for cytochrome c maturation though interacting with the inner mitochondrial membrane (facing the mitochondrial matrix) and forming hetero-couples (Rayapuram et al. 2007, 2008). (3) Trigalactosyldiacylglycerol proteins (TGDs), which have been involved in polar lipid transport (Wang et al. 2012, 2013b), and (4) cobalt transport-associated proteins (CBYs), which have been found to participate in the cobalt transport system (Pang et al. 2013) and unknown function proteins (NOs), which have been suggested to contribute to Al detoxification (Larsen et al. 2005; Huang et al. 2009, 2010).

Plant ABCI subfamily contains only soluble proteins, just like ABCE and ABCF subfamily. But ABCI subfamily is usually shorter in amino acid sequences, because of only one ATP-binding domain. Functions of monocotyledonous ABCIs are poorly understood. There are two genes functionally reported, OsSTAR1 (for sensitive to Al rhizotoxicity1) and OsSTAR2. Disruption of either gene led to hypersensitivity to aluminum toxicity. OsSTAR1 encodes a nucleotide-binding domain and OsSTAR2 encodes a transmembrane domain of the transporter, which are homologous to bacterial-type ABC transporters. Coexpression of OsSTAR1 and OsSTAR2 in *Xenopus laevis* oocytes displayed an efflux activity for UDP-glucose (Huang et al. 2009).

8 Outlook

Genetic and biochemical approaches have revealed a wealth of information on plant ABC transporters, including their structure, regulating factors, and function in plant growth and developmental processes. The range of processes in which members of the eight subfamilies of plant ABC transporters have been implicated encompasses polar auxin transport, alkaloid transport, lipid catabolism, xenobiotic detoxification,

stomatal function, disease resistance, and ion regulation (Martinoia et al. 2002; Rea 2007). However, of the 130 ABC transporters annotated in the Arabidopsis genome, only ~20 have been functionally characterized partially due to the overlapping functions between the members (Kang et al. 2011). The number of members that have been functionally characterized in monocotyledons is much smaller. However, it is reasonable to assume that many monocotyledonous ABC transporters function similar to their Arabidopsis counterparts. On the other side, there are important differences between monocots and dicots, such as fruit structure and pollen formation. Mutation of genes in these functions might uncover of monocot-specific ABC transporter functionalities.

The research on monocotyledonous agricultural crops is relatively difficult but valuable. Overlapping functions of members of plant ABC transporters makes it difficult to reveal real function of the genes when using single mutants. Therefore double or multiple mutants and comparative analysis of gene expression patterns and subcellular localizations of the members in every subfamily will be interesting to pursue in the future. These precious studies then can be applied to future breeding for high-yielding agricultural crops serving human benefits: a member of maize ABCC transporters, ZmMRP4 and its homologous genes in soybean and rice play an essential role in phytic acid accumulation and this study can provide a profitable and environmentally friendly approach to engineer and breed low-phytate varieties. Analogously, a member of wheat ABCG transporters, LR34 was associated with resistance to various wheat diseases and a member of barley ABCG transporters, HvABCG31, is involved in leaf water conservation. These studies can provide an important reference for breeding disease-resistant and drought-resistant varieties.

References

Badone FC, Cassani E, Landoni M et al (2010) The low phytic acid1-241 (lpa1-241) maize mutation alters the accumulation of anthocyanin pigment in the kernel. Planta 231:1189–1199

Bailly A, Sovero V, Vincenzetti V et al (2008) Modulation of P-glycoproteins by auxin transport inhibitors is mediated by interaction with immunophilins. J Biol Chem 28:21817–21826

Bernard DG, Cheng Y, Zhao Y et al (2009) An allelic mutant series of ATM3 reveals its key role in the biogenesis of cytosolic iron-sulfur proteins in Arabidopsis. Plant Physiol 151:590–602

Bessard A, Sole V, Bouchaud G et al (2009) High antitumor activity of RLI, an interleukin-15 (IL-15)-IL-15 receptor alpha fusion protein, in metastatic melanoma and colorectal cancer. Mol Cancer Ther 8:2736–2745

Bessire M, Borel S, Fabre G et al (2011) A member of the PLEIOTROPIC DRUG RESISTANCE family of ATP binding cassette transporters is required for the formation of a functional cuticle in Arabidopsis. Plant Cell 23:1958–1970

Bird D, Beisson F, Brigham A et al (2007) Characterization of Arabidopsis ABCG11/WBC11, an ATP binding cassette (ABC) transporter that is required for cuticular lipid secretion. Plant J 52:485–498

Bisbal C, Silhol M, Laubenthal H et al (2000) The 2'-5' oligoadenylate/RNase L/RNase L inhibitor pathway regulates both MyoD mRNA stability and muscle cell differentiation. Mol Cell Biol 20:4959–4969

Bouchard R, Bailly A, Blakeslee JJ et al (2006) Immunophilin-like TWISTED DWARF1 modulates auxin efflux activities of Arabidopsis P-glycoproteins. J Biol Chem 281:30603–30612
Braz AS, Finnegan J, Waterhouse P et al (2004) A plant orthologue of RNase L inhibitor (RLI) is induced in plants showing RNA interference. J Mol Evol 59:20–30
Cerino Badone F, Amelotti M, Cassani E et al (2012) Study of low phytic acid1-7 (lpa1-7), a new ZmMRP4 mutation in maize. J Hered 103:598–605
Chen G, Sagi M, Weining S et al (2004) Wild barley eibi1 mutation identifies a gene essential for leaf water conservation. Planta 219:684–693
Chen ZQ, Dong J, Ishimura A et al (2006) The essential vertebrate ABCE1 protein interacts with eukaryotic initiation factors. J Biol Chem 281:7452–7457
Chen S, Sánchez-Fernández R, Lyver ER et al (2007) Functional characterization of AtATM1, AtATM2, and AtATM3, a subfamily of Arabidopsis half-molecule ATP-binding cassette transporters implicated in iron homeostasis. J Biol Chem 282:21561–21571
Chen G, Komatsuda T, Pourkheirandish M et al (2009a) Mapping of the eibi1 gene responsible for the drought hypersensitive cuticle in wild barley (Hordeum spontaneum). Breed Sci 59:21–26
Chen G, Pourkheirandish M, Sameri M et al (2009b) Genetic targeting of candidate genes for drought sensitive gene eibi1 of wild barley (Hordeum spontaneum). Breed Sci 59:637–644
Chen G, Komatsuda T, Ma JF et al (2011) An ATP-binding cassette subfamily G full transporter is essential for the retention of leaf water in both wild barley and rice. Proc Natl Acad Sci U S A 108:12354–12359
Cho M, Lee SH, Cho HT (2007) P-glycoprotein4 displays auxin efflux transporter-like action in Arabidopsis root hair cells and tobacco cells. Plant Cell 19:3930–3943
Choi H, Jin JY, Choi S et al (2011) An ABCG/WBC-type ABC transporter is essential for transport of sporopollenin precursors for exine formation in developing pollen. Plant J 65:181–193
Christie JM, Yang H, Richter GL et al. (2011) phot1 inhibition of ABCB19 primes lateral auxin fluxes in the shoot apex required for phototropism. PLoS Biol 9(6):e1001076.
Davies TGE, Theodoulou FL, Hallahan DL et al (1997) Cloning and characterisation of a novel p-glycoprotein homologue from barley. Gene 199:195–202
Dietrich D, Schmuths H, De Marcos LC et al (2009) Mutations in the Arabidopsis peroxisomal ABC transporter COMATOSE allow differentiation between multiple functions in planta: insights from an allelic series. Mol Biol Cell 20:530–543
Dou XY, Yang KZ, Zhang Y et al (2011) WBC27, an adenosine tri-phosphate-binding cassette protein, controls pollen wall formation and patterning in Arabidopsis. J Integr Plant Biol 53:74–88
Footitt S, Slocombe SP, Larner V et al (2002) Control of germination and lipid mobilization by COMATOSE, the Arabidopsis homologue of human ALDP. EMBO J 21:2912–2922
Garcia O, Bouige P, Forestier C et al (2004) Inventory and comparative analysis of rice and Arabidopsis ATP-binding cassette (ABC) systems. J Mol Biol 343:249–265
Geisler M, Kolukisaoglu HU, Bouchard R et al (2003) TWISTED DWARF1, a unique plasma membrane-anchored immunophilin-like protein, interacts with Arabidopsis multidrug resistance-like transporters AtPGP1 and AtPGP19. Mol Biol Cell 14:4238–4249
Geisler M, Girin M, Brandt S et al (2004) Arabidopsis immunophilin-like TWD1 functionally interacts with vacuolar ABC transporters. Mol Biol Cell 15:3393–3405
Geisler M, Blakeslee JJ, Bouchard R et al (2005) Cellular efflux of auxin catalyzed by the Arabidopsis MDR/PGP transporter AtPGP1. Plant J 44:179–194
Goodman CD, Casati P, Walbot V (2004) A multidrug resistance—associated protein involved in anthocyanin transport in *Zea mays*. Plant Cell 16:1812–1826
Hayashi M, Nito K, Takei-Hoshi R et al (2002) Ped3p is a peroxisomal ATP-binding cassette transporter that might supply substrate for fatty acid β-oxidation. Plant Cell Physiol 43:1–11
Henrichs S, Wang B, Fukao Y et al (2012) Regulation of ABCB1/PGP1-catalysed auxin transport by linker phosphorylation. EMBO J 31:2965–2980
Hettema EH, Tabak HF (2000) Transport of fatty acids and metabolites across the peroxisomal membrane. Biochim Biophys Acta 1486:18–27

Higgins CF, Linton KJ (2004) The ATP switch model for ABC transporters. Nat Struct Mol Biol 11:918–926

Hooks MA, Turner JE, Murphy EC et al (2007) The Arabidopsis ALDP protein homologue COMATOSE is instrumental in peroxisomal acetate metabolism. Biochem J 406:399–406

Huang CF, Yamaji N, Mitani N et al (2009) A bacterial-type ABC transporter is involved in aluminum tolerance in rice. Plant Cell 21:655–667

Huang CF, Yamaji N, Ma JF (2010) Knockout of a bacterial-type ABC transporter gene, AtSTAR1, results in increased Al sensitivity in Arabidopsis. Plant Physiol 153:1669–1677

Jasinski M, Ducos E, Martinoia E et al (2003) The ATP-binding cassette transporters: structure, function, and gene family comparison between rice and Arabidopsis. Plant Physiol 131:1169–1177

Kamimoto Y, Terasaka K, Hamamoto M et al (2012) Arabidopsis ABCB21 is a facultative auxin importer/exporter regulated by cytoplasmic auxin concentration. Plant Cell Physiol 53:2090–2100

Kang J, Hwang JU, Lee M et al (2010) PDR-type ABC transporter mediates cellular uptake of the phytohormone abscisic acid. Proc Natl Acad Sci U S A 107:2355–2360

Kang J, Park J, Choi H et al (2011) Plant ABC transporters. Arabidopsis Book 9:e0153

Karcher A, Buttner K, Martens B et al (2005) X-ray structure of RLI, an essential twin cassette ABC ATPase involved in ribosome biogenesis and HIV capsid assembly. Structure 13:649–659

Kim DY, Bovet L, Kushnir S et al (2006) AtATM3 is involved in heavy metal resistance in Arabidopsis. Plant Physiol 140:922–932

Kim DY, Bovet L, Maeshima M et al (2007) The ABC transporter AtPDR8 is a cadmium extrusion pump conferring heavy metal resistance. Plant J 50:207–218

Klein M, Burla B, Martinoia E (2006) The multidrug resistance-associated protein (MRP/ABCC) subfamily of ATP-binding cassette transporters in plants. FEBS Lett 580:1112–1122

Knoller AS, Blakeslee JJ, Richards EL et al (2010) Brachytic2/*Zm*ABCB1 functions in IAA export from intercalary meristems. J Exp Bot 61:3689–3696

Kobae Y, Sekino T, Yoshioka H et al (2006) Loss of AtPDR8, a plasma membrane ABC transporter of *Arabidopsis* thaliana, causes hypersensitive cell death upon pathogen infection. Plant Cell Physiol 47:309–318

Kou X, Watkins CB, Gan SS (2012) Arabidopsis AtNAP regulates fruit senescence. J Exp Bot 63:6139–6147

Krattinger SG, Lagudah ES, Spielmeyer W et al (2009) A putative ABC transporter confers durable resistance to multiple fungal pathogens in wheat. Science 323:1360–1363

Kubeš M, Yang H, Richter GL et al (2012) The Arabidopsis concentration-dependent influx/efflux transporter ABCB4 regulates cellular auxin levels in the root epidermis. Plant J 69:640–654

Kuromori T, Miyaji T, Yabuuchi H et al (2010) ABC transporter AtABCG25 is involved in abscisic acid transport and responses. Proc Natl Acad Sci U S A 107:2361–2366

Kuromori T, Sugimoto E, Shinozaki K (2011) Arabidopsis mutants of AtABCG22, an ABC transporter gene, increase water transpiration and drought susceptibility. Plant J 67:885–894

Larsen PB, Geisler MJ, Jones CA et al (2005) ALS3 encodes a phloem-localized ABC transporter-like protein that is required for aluminum tolerance in Arabidopsis. Plant J 41:353–363

Larsen PB, Cancel J, Rounds M et al (2007) Arabidopsis ALS1 encodes a root tip and stele localized half type ABC transporter required for root growth in an aluminum toxic environment. Planta 225:1447–1458

Lee M, Choi Y, Burla B et al (2008) The ABC transporter AtABCB14 is a malate importer and modulates stomatal response to CO_2. Nat Cell Biol 10:1217–1223

Lewis DR, Miller ND, Splitt BL et al (2007) Separating the roles of acropetal and basipetal auxin transport on gravitropism with mutations in two Arabidopsis multidrug resistance-like ABC transporter genes. Plant Cell 19:1838–1850

Li ZS, Zhao Y, Rea PA (1995) Magnesium adenosine 5'-triphosphate-energized transport of glutathione S-conjugates by plant vacuolar membrane vesicles. Plant Physiol 107:1257–1268

Liu ZY, Jacobs M, Schaff DA et al (2001) ChvD, a chromosomally encoded ATP-binding cassette transporter-homologous protein involved in regulation of virulence gene expression in Agrobacterium tumefaciens. J Bacteriol 183:3310–3317
Lu YP, Li ZS, Rea PA (1997) *AtMRP1* gene of *Arabidopsis* encodes a glutathione S-conjugate pump: isolation and functional definition of a plant ABC-binding cassette transporter gene. Proc Natl Acad Sci U S A 84:8243–8248
Lu YP, Li ZS, Drozdowicz YM et al (1998) AtMRP2, an Arabidopsis ATP-binding cassette transporter able to transport glutathione S-conjugates and chlorophyll catabolites: functional comparison with AtMRP1. Plant Cell 10:267–282
Maathuis FJ, Filatov V, Herzyk P et al (2003) Transcriptome analysis of root transporters reveals participation of multiple gene families in the response to cation stress. Plant J 35:675–692
Marrs KA, Alfenito MR, Lloyd AM et al (1995) A glutathione S-transferase involved in vacuolar transfer encoded by the maize gene Bronze-2. Nature 375:397–400
Martinoia E, Grill E, Tommasini R et al (1993) ATP-dependent glutathione S-conjugate "export" pumps in the vacuolar membrane of plants. Nature 364:247–249
Martinoia E, Klein M, Geisler M et al (2002) Multifunctionality of plant ABC transporters-more than just detoxifiers. Planta 214:345–355
Marton MJ, Vazquez de Aldana CR, Qiu H et al (1997) Evidence that GCN1 and GCN20, translational regulators of GCN4, function on elongating ribosomes in activation of eIF2 alpha kinase GCN2. Mol Cell Biol 17:4474–4489
Matsuda S, Kajizuka T, Kadota A et al (2011) NPH3- and PGP-like genes are exclusively expressed in the apical tip region essential for blue-light perception and lateral auxin transport in maize coleoptiles. J Exp Bot 62:3459–3466
Mentewab A, Stewart CN Jr (2005) Overexpression of an Arabidopsis thaliana ABC transporter confers kanamycin resistance to transgenic plants. Nat Biotechnol 23:1177–1180
Moons A (2003) Ospdr9, which encodes a PDR-type ABC transporter, is induced by heavy metals, hypoxic stress and redox perturbations in rice roots. FEBS Lett 553:370–376
Moons A (2008) Transcriptional profiling of the PDR gene family in rice roots in response to plant growth regulators, redox perturbations and weak organic acid stresses. Planta 229:53–71
Multani DS, Briggs SP, Chamberlin MA et al (2003) Loss of an MDR transporter in compact stalks of maize br2 and sorghum dw3 mutants. Science 302:81–84
Noh B, Murphy AS, Spalding EP (2001) Multidrug resistance-like genes of Arabidopsis required for auxin transport and auxin-mediated development. Plant Cell 13:2441–2454
Nyathi Y, De Marcos LC, van Roermund CW et al (2010) The Arabidopsis peroxisomal ABC transporter, comatose, complements the Saccharomyces cerevisiae pxa1 pxa2Δ mutant for metabolism of long-chain fatty acids and exhibits fatty acyl-CoA-stimulated ATPase activity. J Biol Chem 285:29892–29902
Oda K, Otani M, Uraguchi S et al (2011) Rice ABCG43 is Cd inducible and confers Cd tolerance on yeast. Biosci Biotechnol Biochem 75:1211–1213
Pang K, Li Y, Liu M et al (2013) Inventory and general analysis of the ATP-binding cassette (ABC) gene superfamily in maize (Zea mays L.). Gene 526:411–428
Panikashvili D, Savaldi-Goldstein S, Mandel T et al (2007) The Arabidopsis DESPERADO/AtWBC11 transporter is required for cutin and wax secretion. Plant Physiol 145:1345–1360
Panikashvili D, Shi JX, Bocobza S et al (2010) The Arabidopsis DSO/ABCG11 transporter affects cutin metabolism in reproductive organs and suberin in roots. Mol Plant 3:563–575
Panikashvili D, Shi JX, Schreiber L et al (2011) The Arabidopsis ABCG13 transporter is required for flower cuticle secretion and patterning of the petal epidermis. New Phytol 190:113–124
Pighin JA, Zheng H, Balakshin LJ et al (2004) Plant cuticular lipid export requires an ABC transporter. Science 306:702–704
Qin P, Tu B, Wang Y et al (2013) ABCG15 encodes an ABC transporter protein, and is essential for post-meiotic anther and pollen exine development in rice. Plant Cell Physiol 54:138–154

Quilichini TD, Friedmann MC, Samuels AL et al (2010) ATP-binding cassette transporter G26 is required for male fertility and pollen exine formation in Arabidopsis. Plant Physiol 154:678–690

Raboy V (2007) The ABCs of low-phytate crops. Nat Biotechnol 25:874–875

Rayapuram N, Hagenmuller J, Grienenberger et al (2007) AtCCMA interacts with AtCcmB to form a novel mitochondrial ABC transporter involved in cytochrome c maturation in Arabidopsis. J Biol Chem 282:21015–21023

Rayapuram N, Hagenmuller J, Grienenberger JM et al (2008) The three mitochondrial encoded CcmF proteins form a complex that interacts with CCMH and c-type apocytochromes in Arabidopsis. J Biol Chem 283:25200–25208

Rea PA (2007) Plant ATP-binding cassette transporters. Annu Rev Plant Biol 58:347–375

Rensing SA, Lang D, Zimmer AD et al (2008) The Physcomitrella genome reveals evolutionary insights into the conquest of land by plants. Science 319:64–69

Russell L, Larner V, Kurup S et al (2000) The Arabidopsis COMATOSE locus regulates germination potential. Development 127:3759–3767

Sasaki T, Ezaki B, Matsumoto H (2002) A gene encoding multidrug resistance (MDR) -like protein is induced by aluminum and inhibitors of calcium flux in wheat. Plant Cell Physiol 43:177–185

Shen C, Bai Y, Wang S et al (2010) Expression profile of PIN, AUX/LAX and PGP auxin transporter gene families in Sorghum bicolor under phytohormone and abiotic stress. FEBS Lett 277:2954–2969

Shi J, Wang H, Schellin K et al (2007) Embryo-specific silencing of a transporter reduces phytic acid content of maize and soybean seeds. Nat Biotechnol 25:930–937

Sidler M, Hassa P, Hasan S et al (1998) Involvement of an ABC transporter in a developmental pathway regulating hypocotyl cell elongation in the light. Plant Cell 10:1623–1636

Terasaka K, Blakeslee JJ, Titapiwatanakun B et al (2005) PGP4, an ATP binding cassette P glycoprotein, catalyzes auxin transport in Arabidopsis thaliana roots. Plant Cell 17:2922–2939

Teschner J, Lachmann N, Schulze J et al (2010) A novel role for Arabidopsis mitochondrial ABC transporter ATM3 in molybdenum cofactor biosynthesis. Plant Cell 22:468–480

Theodoulou FL, Job K, Slocombe SP et al (2005) Jasmonic acid levels are reduced in COMATOSE ATP-binding cassette transporter mutants. Implications for transport of jasmonate precursors into peroxisomes. Plant Physiol 137:835–840

Tillett D, Dittmann E, Erhard M et al (2000) Structural organization of microcystin biosynthesis in Microcystis aeruginosa PCC7806: an integrated peptide-polyketide synthetase system. Chem Biol 7:753–764

van den Brule S, Muller A, Fleming AJ et al (2002) The ABC transporter SpTUR2 confers resistance to the antifungal diterpene sclareol. Plant J 30:649–662

Vasiliou V, Vasiliou K, Nebert DW (2009) Human ATP-binding cassette (ABC) transporter family. Hum Genomics 3:281–290

Verrier PJ, Bird D, Burla B et al (2008) Plant ABC proteins–a unified nomenclature and updated inventory. Trends Plant Sci 13:151–159

Wanders RJ, Visser WF, van Roermund CW et al (2007) The peroxisomal ABC transporter family. Pflugers Arch 453:719–734

Wang Z, Xu C, Benning C (2012) TGD4 involved in endoplasmic reticulum-to-chloroplast lipid trafficking is a phosphatidic acid binding protein. Plant J 70:614–623

Wang B, Bailly A, Zwiewka M et al (2013a) Arabidopsis TWISTED DWARF1 functionally interacts with auxin exporter ABCB1 on the root plasma membrane. Plant Cell 25:202–214

Wang Z, Anderson NS, Benning C (2013b) The phosphatidic acid binding site of the Arabidopsis trigalactosyldiacylglycerol 4 (TGD4) protein required for lipid import into chloroplasts. J Biol Chem 288:4763–4771

Winans SC, Kerstetter RA, Nester EW (1988) Transcriptional regulation of the virA and virG genes of Agrobacterium tumefaciens. J Bacteriol 170:4047–4054

Wu G, Lewis DR, Spalding EP (2007) Mutations in Arabidopsis multidrug resistance-like ABC transporters separate the roles of acropetal and basipetal auxin transport in lateral root development. Plant Cell 19:1826–1837

Wu G, Otegui MS, Spalding EP (2010) The ER-localized TWD1 immunophilin is necessary for localization of multidrug resistance-like proteins required for polar auxin transport in Arabidopsis roots. Plant Cell 22:3295–3304

Xi J, Xu P, Xiang CB (2012) Loss of AtPDR11, a plasma membrane-localized ABC transporter, confers paraquat tolerance in Arabidopsis thaliana. Plant J 69:782–791

Xu XH, Zhao HJ, Liu QL et al (2009) Mutations of the multi-drug resistance-associated protein ABC transporter gene 5 result in reduction of phytic acid in rice seeds. Theor Appl Genet 119:75–83

Yasuno N, Takamure I, Kidou S et al (2009) Rice shoot branching requires an ATP-binding cassette subfamily G protein. New Phytol 182:91–101

Zimmerman C, Klein KC, Kiser PK et al (2002) Identification of a host protein essential for assembly of immature HIV-1 capsids. Nature 415:88–92

Zolman BK, Silva ID, Bartel B (2001) The Arabidopsis pxa1 mutant is defective in an ATP-binding cassette transporter-like protein required for peroxisomal fatty acid β-oxidation. Plant Physiol 127:1266–1278

Structure–Function of Plant ABC-Transporters

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Abstract The ABC-transporter protein superfamily assures the crossmembrane transport of a broad variety of substrates in all living forms. In plants, they contribute greatly to most of the cellular functions, ranging from nutrient exchange to secondary metabolites or hormonal control. This transport mechanism is solely energized by the consumption of two ATP molecules per transport cycle. The elegant transmission interface linking the ATP hydrolysis to the selective translocation of solute across lipid layers was recently revealed thanks to structural information gained by crystallography studies. The simple modular organization of these transporters and the degree of homology between them argue for a conserved association of a functional RecA-/F1-ATPase-like nucleotide-binding dimer providing the energy stroke sufficient to alternatively reorganize a transmembrane module that selects and vectorially drives solutes. The helices forming this transmembrane domain essentially create a pore with successive opening and closing sequences exhibiting different substrate affinities at each side of the membrane. Therefore, the surface residues forming this translocation chamber define the function of each transporter and their careful analysis may prove useful to elucidate the ABC-machinery. The need to understand the way these proteins function is not only driven by clinical issues such as the multidrug resistance phenomenon but as well by the promise to control key cellular events in diverse organisms. Manipulation of plant genes altering ACB-transporters functions has already brought encouraging perspectives in miscellaneous research areas from phytoremediation to plant architecture modification.

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1 Selective Molecular Pumps

ATP-binding cassette (ABC) transporters are universally found in all phyla, from prokaryotes to humans (Higgins 1992; Hrycyna and Gottesman 1998), and constitute one of the largest and most conserved protein superfamilies (Holland and Blight 1999; Isenbarger et al. 2008; Holland 2011). These integral proteins function in the vectorial translocation of substrates across intra- and extracellular membranes and are believed to utilize ATP binding and hydrolysis to energize the conformational changes required for this transport mechanism. Decades of focused research have revealed the astonishing diversity of tasks imputed to ABC-transporters within the life cycle of organisms, ranging from nutrient uptake, lipid trafficking, and maintenance of osmotic homeostasis to cell division and development (Higgins 1992; Holland and Blight 1999; Martinoia et al. 2002; Kretzschmar et al. 2011; Dean and Annilo 2005). Typically these functions are achieved through the specific transport of substrates inferring that ABC-transporters hold a certain degree of specificity in the recognition of their ligands. However, most of the efforts to characterize the structure-to-function relationships inside this large protein family have been driven by the urge to elucidate major human health issues. Among those a subset of human ABC-transporters, namely ABCB1 (P-glycoprotein/Multi Drug Resistance 1), ABCC1 (Multidrug Resistance-associated Protein 1) and ABCG2 (Breast Cancer Resistance Protein) have been associated with the multidrug resistance (MDR) phenotype in cancer cells that developed resistance to chemotherapeutic drugs (Sharom 2008; Holland 2011). Thereafter, ABC-transporters have been viewed through the prism of MDR despite the fact that the broad ligand specificity exerted by MDR-associated proteins and their apparent role in extruding xenobiotics does not accurately portray the diverse family members.

The growing number of characterized microbial, animal, and plant ABC-transporters, both at the molecular and biochemical levels, has on one hand continuously unveiled their functional variety but, on the other hand, revealed a common protein structural design (Kretzschmar et al. 2011; Verrier et al. 2008; Dean and Annilo 2005). Given the large number of ABC genes present in living organisms (e.g., 65 in *E. coli*, 57 in *Drosophila*, 48 in human, and 120 in *Arabidopsis*), one can wonder how these conserved structural features accommodate diverse ligand selectivity.

To date, there is no structural data available specific for plant ABC architectures. The difficult task to crystallize these large membrane proteins has been slowing our understanding of their mechanism for decades and each newly reported structure generates a broad enthusiasm for the interpretation of former datasets and the formulation of new mechanistic concepts or evolutionary perspectives. In the recent years crystallographic data eventually exposed the full structure of key ABC transporters. Besides prokaryote-specific ABC importers greatly helped in appreciating the subtlety of the molecular events driving the consumption of ATP energy to the coupling of the allosteric transport coordination. Two particular milestones have

cast light onto eukaryotic, and indirectly also onto plant, structural ABC design. Publication of the *S. aureus* Sav1866 (Dawson and Locher 2006) and of the murine multidrug exporter ABCB1/MDR1A (Aller et al. 2009) provided a framework for analyzing the central specialization of plant ABC transporters to their substrates.

The need in a reliable system to address nutrients or secondary metabolites to the desired cellular compartments reflects the sessile habit of these organisms. Given the absence of a central nervous system based on rapid electric pulses, plants developed a very elegant chemical communication apparatus to timely and precisely deliver the information. This is then no surprise that plant ABC-transporters participate in critical processes in growth and development, from hormonal control to plant architecture achievement (Martinoia et al. 2002; Kretzschmar et al. 2011). The strong substrate-specificity expressed by plant ABC-proteins is therefore a great asset to study the structure-to-function relationships of the transport cycle of such pumps (Geisler et al. 2005). Plant ABC-transporters display sufficient sequence homology to the published crystallized proteins to attempt robust in silico modeling and further analysis of the spatial distribution of key residues in the ACB catalytic cycle (Kretzschmar et al. 2011; Bailly et al. 2012).

2 A Millions-Year Core Architecture

The core architecture of ABC-transporters consists of a conserved arrangement of two transmembrane domains (TMDs) and two cytosolic nucleotide-binding domains (NBDs, ATP-binding cassettes). Functional transporters can be encoded by one or several discrete genes, translated into one, two or four polypeptides which eventually assemble into mono-, di-, or tetramers (Fig. 1). Prokaryotic ABC-importers comprise a supplementary periplasmic-binding protein that captures the targeted substrate from the extracellular environment and delivers it to the TMDs, thus reflecting their prevailing function in primary metabolites uptake. Early studies demonstrating the involvement of eukaryotic ABC-transporters in detoxification processes led to the concept that these proteins exclusively extrude substrates from the cytosol. However, eukaryotic ABC-transporters are found in most cell membranes (Dean and Annilo 2005; Kretzschmar et al. 2011) and at least two plant ABC-transporters members of the B-subfamily can also act as importers, referred to as facultative im/exporters (see Chap. 14 Evolution of transport directionality in ABCBs, Shitan et al. 2003; Kubes et al. 2011). To date, it is not known if this particular import mechanism can be extended to other ABC-exporters, so we will cautiously hereafter use the term *exporter* to distinguish these proteins from the prokaryotic ABC-importer type. Yet, this import mechanism is not incompatible with the prevailing concept of ABC-transport mechanism in eukaryotes.

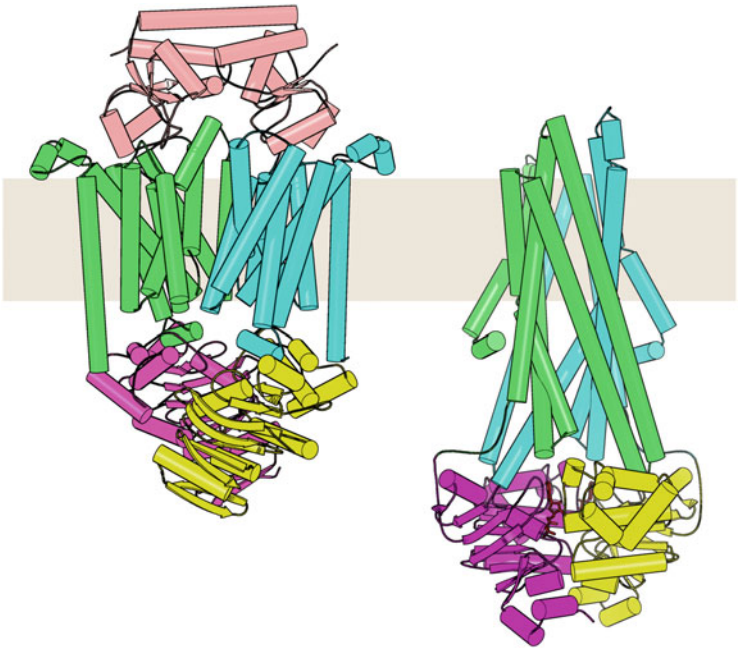

Fig. 1 Canonical ABC-transporter architectures. ABC-importers (*left*) and exporters (*right*) share a common structural organization. In their ATP-bound state, the two nucleotide-binding domains (*in purple and yellow*) form a tight dimer serving as a socket for the pack of α-helices forming the transmembrane domains (*in green and blue*) open to the outer medium. In this state, exporters achieve the extrusion of solutes, while importers accept the uptake substrate from the periplasmic-binding protein (*in pink*)

2.1 The Nucleotide-Binding Fold: A Universal Engine Fueled with Universal Gas

Original crystal structures showed that soluble NBDs in the ATP-bound state formed a closed head-to-tail dimer with two nucleotides bound at its interface (Fig. 2), while nucleotide-free NBDs were spatially disconnected. From these observations emerged the so-called *switch model* in which each NBD monomer binds a molecule of ATP leading to a closed NBD dimer and consecutive hydrolysis of two nucleotides (Higgins and Linton 2004; Holland and Blight 1999). Posthydrolysis, the NBD monomers separate and release ADP and Pi. The exact timing of ATP binding to each NBD is yet not entirely clear and both possibilities of simultaneous or alternative binding of the nucleotides are currently debated (Jones and George 2013; George and Jones 2012; Hollenstein et al. 2007). Nonetheless, it is assumed that cyclic opening and closing of the NBD dimer drives the conformational changes in TMDs that grant substrate translocation. The collection of nucleotide-bound or -free soluble NBD dimers and full ABC-transporters structures

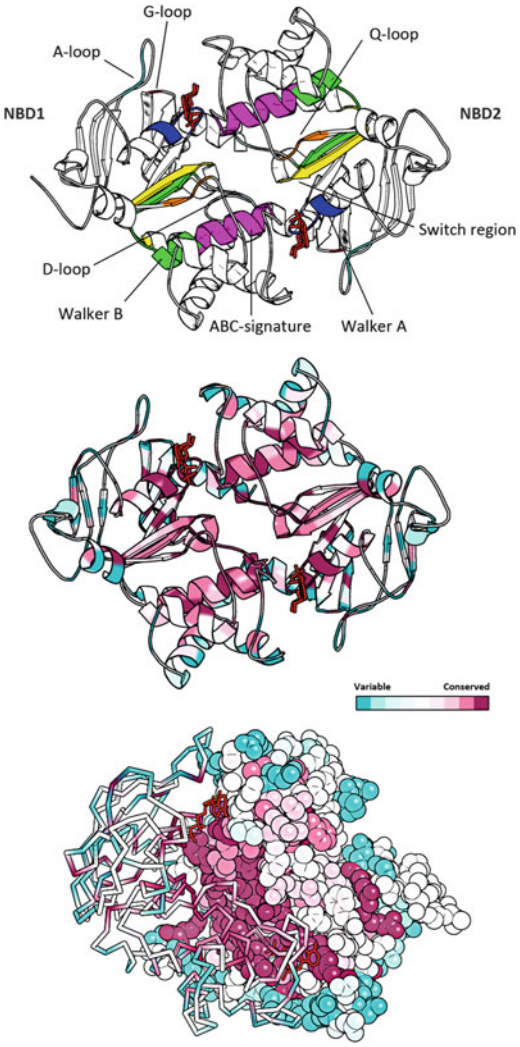

Fig. 2 The nucleotide-binding fold: a conserved life energizer. (**a**) Pseudosymmetry of the NBD dimer in the nucleotide-bound state. The principal functional segments participating in ATP-binding and hydrolysis are highlighted in discrete colors. Nucleotides are represented as *red sticks*. (**b**) Detailed residue conservation status of the NBD dimer. Note the high conservation of signature motifs at the nucleotide interface. Conservation scores were calculated based on the positional sequence alignment of 21 different prokaryotic and eukaryotic NDB crystal structures using the ConSurf server (http://consurf.tau.ac.il). (**c**) Same as (**b**), one NBD monomer is represented as ribbons and the opposite monomer as spheres. The NBD dimer was slightly tilted to visualize the remarkable conservation status of the NBD–NBD interface. In contrast, residues exposed to the hydrophilic medium display high variability

available to date has questioned this canonical view (Jones et al. 2009), although without completely rejecting it. Indeed, the various degrees of separation between NBD monomers observed in these crystal data are probably incompatible with a model where the sole energy of ATP binding would be sufficient to bring the two halves of the sandwich dimer together (Jones et al. 2009; Jones and George 2013; George and Jones 2012; Holland 2011; Verhalen and Wilkens 2011). Thus, until further evidence, it appears reasonable to hypothesize that, in its cellular context, the structural movements between the open and closed NBD dimers of an integral ABC-transporter are probably restrained to the lowest energy cost.

It is important to state here that plant NBDs are not much different from other kingdom NBDs and apparently perform the same described ancestral function (Isenbarger et al. 2008). In regard to the essential motifs to perform ABC catalytic cycle, linear alignments of plant ABC-transporter polypeptides clearly show a high functional conservation (Fig. 2). Typically, each NBD can be subdivided into two subdomains. The alpha-beta subdomain 1 includes a RecA-/F1-ATPase-like nucleotide-binding region comprising the Walker A (or P-loop, GXXGXGKS/T) and Walker B (JJJJDE) motifs (X = any and J = aliphatic residue) common to all ATP-binding proteins and a characteristic ABC-ATPase beta-subdomain. The α-helical subdomain 2 contains the ABC-signature motif (LSGGQ) and displays a flexible attachment to subdomain 1. The head-to-tail orientation of the NBD dimer defines two composite sites for ATP binding formed by the Walker A and Walker B motifs of one monomer and the ABC-signature stretch of the other monomer. In addition to these motifs, several functionally conserved residues coordinately participate in the binding and hydrolysis of ATP. Although the exact mechanism of ATP hydrolysis is still controversial (George and Jones 2012; Jones et al. 2009), the following events are supported by biochemical and structural evidence: (1) a conserved aromatic residue in the A-loop preceding the Walker A motif builds a ring-stacking interaction with the adenosine ring of the nucleotide, thus positioning it in the catalytic site; (2) residues from the Walker A motif, especially the highly conserved lysine, form hydrogen bonds with the oxygen atoms of ATP β- and γ-phosphates further coordinated with waters and a Mg^{2+} ion; (3) in addition, the Walker B conserved aspartate forms hydrogen bonds to the catalytic Mg^{2+}ion, thus supporting the geometry of the catalytic site; (4) backbone amide groups of glycine and side chain of serine residues from the ABC-signature motif drive a positive charge dipole to the nucleotide γ-phosphate, in analogy to interdomain arginine fingers found in other P-loop ATPases; (5) the conserved histidine contained in the Switch region (or H-loop) as well interacts with the nucleotide γ-phosphate and the opposite Walker A and D-loop (SALD) glutamate following the Walker B motif. Therefore the Switch region and D-loop stretches represent the interface of the tight coupling of nucleotide binding and NBD dimerization and may exert a regulatory function in the catalytic site. Conformational changes in these regions may alter the orientation of critical residues in and affect communication between the active sites. Indeed, once nucleotide phosphates are properly bound and its γ-phosphate presented to the attacking water and coordinating Mg^{2+}, a catalytic base is required to favor the hydrolytic reaction.

The Walker B terminal glutamate and the Switch region histidine are currently seen as favorite candidates, but the conserved glutamine defining the Q-loop stretch that connects the ATP core and α-subdomains could potentially perform this role.

It is now clear that substrate import or export across the TMDs is allosterically coupled to ATP binding/hydrolysis, though a detailed mechanism is still pending (Jones and George 2013; Procko et al. 2009). The Q-loop participates in the interface with the TMDs and conformational changes in this region during the catalytic cycle and could thus potentially engage its conserved glutamine in the hydrolytic active site or disengage it following ATP hydrolysis. Such a movement could be involved in transmitting structural changes between TMDs and NBDs via the coupling helices described hereafter. A second exporter-specific feature that may participate in NBD–TMD transmission interface is the presence of a conserved glutamate from the X-loop motif in each NBD, shown to cross-contact the TMD coupling helices in the nucleotide-bound state (Hollenstein et al. 2007; Dawson and Locher 2006). While the ultraconserved NBD engines propel and presumably control the transport cycle, they do not directly participate in substrate recognition. This task is sustained by the less obvious structural function of the TMDs.

2.2 The Transmembrane Domains Build Selective Translocation Chambers

The TMDs comprise cytosolic and membrane spanning regions and define the solvent-filled transmembrane aperture containing the substrate-binding sites. The TMD helices considerably extend further than the predicted membrane spans into the cytoplasm and these cytosolic regions, referred to as intracytoplasmic loops (ICLs, Fig. 3), describe a physical interface between TMDs and NBDs believed to couple ATP binding/hydrolysis with the recognition of substrates and their translocation (Jones and George 2013; Hollenstein et al. 2007).

The canonical arrangement of TMDs is of two modules of six hydrophobic transmembrane helices within ABC-transporters, but this number can vary in ABC-importer types and the ABCC/Multidrug resistance-associated protein transporters family (Klein et al. 2006; Tusnady et al. 2006; Kretzschmar et al. 2011) that exhibits a supplementary N-terminal module termed TMD0. The function of this additional transmembrane domain is to date unknown in plant transporters but was suggested to be involved in protein targeting (Mason and Michaelis 2002; Westlake et al. 2005) and could therefore be linked to the exclusive vacuolar localization of these proteins. However, TMDs are loosely conserved in sequence and length and this apparent diversification could represent the rationale for their diverse substrate specificity. Indeed, in functional ABC-transporters the two TMD modules form a gated translocation chamber that alternatively separate each side of the membrane during the catalytic cycle, thus preventing passive diffusion of substrates (Fig. 4; Bailly et al. 2012).

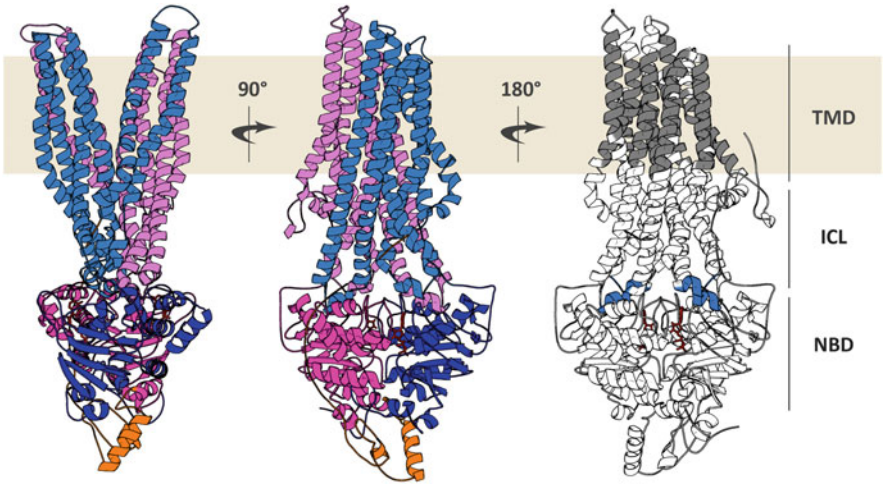

Fig. 3 Cartoon representation of an ABCB homology model based on the Sav1866 outward-facing structure 2HYD. Each covalently bound NBD–TMD module is highlighted in similar colors. In silico prediction of the linker domain bridging the two modules is highlighted *in orange*. *Right*, solely the software-predicted membrane- spanning regions (*black*) and coupling helices (*blue*) are emphasized for clarity. Nucleotides are represented as *red sticks*

Currently available ABC-transporter crystal structures display transmembrane folds with apparent structural discrepancies (Jones et al. 2009, Fig. 5). Obviously, bacterial importers show large helix bundles that form either wide or narrow chambers within the membrane and differ greatly from the TMD arrangements in exporters. Structures from the *Staphylococcus aureus* multidrug exporter Sav1866 (Dawson and Locher 2006), the *E. coli*MsbA lipid flippase (Ward et al. 2007), the mouse multidrug exporter ABCB1/MDR1A (Aller et al. 2009), the *C. elegans* ABCB1/MDR1 (Jin et al. 2012) and the human mitochondrial exporter ABCB10 (Shintre et al. 2013) each exhibit the double six transmembrane helices arrangement forming large V-shaped funnels in both outward- and inward-facing conformations (Fig. 5). These exporters are close and functionally related homologs and represent to date the only basis to apprehend the structural design of eukaryotic ABC-transporters.

It is generally expected that ABC-transporters have evolved from a common ancestor protein (Isenbarger et al. 2008; Verrier et al. 2008; Fisher et al. 2012) with maintained general structure and mechanism that developed to accommodate different substrates. The diverse transmembrane structures obtained through crystallographic studies suggest distinct spatial constraints to embed these membrane-spanning regions into lipid bilayers but phylogenetic classification of TMDs revealed good correlations between linear sequences. It was as well established that the bacterial importers' architecture separated from exporter-like architectures in a single event before the separation between prokaryotic and eukaryotic organisms (Saurin et al. 1999). Therefore, it is tempting to assume that evolution has

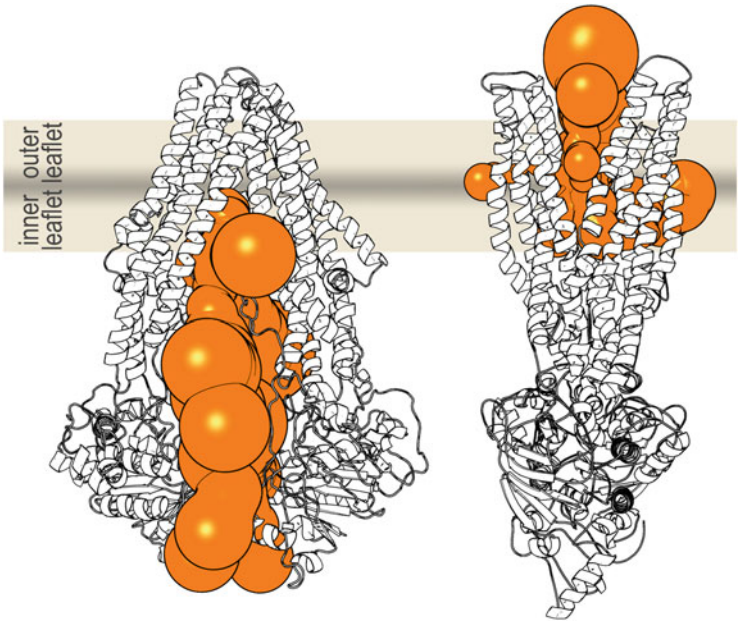

Fig. 4 The transmembrane helices packing in the inward-facing (*left*) and outward-facing (*right*) conformations of ABC-exporters define a solvent-accessible space (*orange volumes*) respectively granting exclusive entrance and exit to substrates across the membrane bilayer. Solvent access paths were calculated using the Mole software (http://mole.chemi.muni.cz)

favored adaptation to novel substrate uptake or export functions from pre-existing, efficient mechanisms. The size of particular TMD sets does not fully correlate with transport specificity; obviously bulky substrates should be accommodated in translocation chambers large enough to sterically accept the molecules (Locher et al. 2002). But functional analysis of transporters, especially plant exporters, revealed that closely related transmembrane pores can translocate substrates of various dimensions (Bikadi et al. 2011; Gutmann et al. 2010; Geisler et al. 2005; Bouchard et al. 2006). Moreover, the lengths of TMDs seem to play no critical role in bacterial ABC-uptake since the periplasmic-binding protein selects for the imported substrate. The key for ligand recognition probably lies then within the intrinsic distribution of residues defining the walls of each TMD cavity.

Despite growing collections of ABC-type sequences obtained from a large spectrum of species it is to date practically impossible to *de novo* predict a function for a given transporter. The reverse analysis, namely defining a substrate-recognition area within the translocation chamber for a known ligand, remains also very challenging. Most of the work conducted so far with metadata from linear sequences and crystallographic coordinates has been restricted to phylogenetic reconstruction and confirmation of previously obtained biological evidence, mainly in the scope of MDR (Procko et al. 2009; Sharom 2008). The difficulties are manifold: first, the number of ABC-transporter genes without assigned function

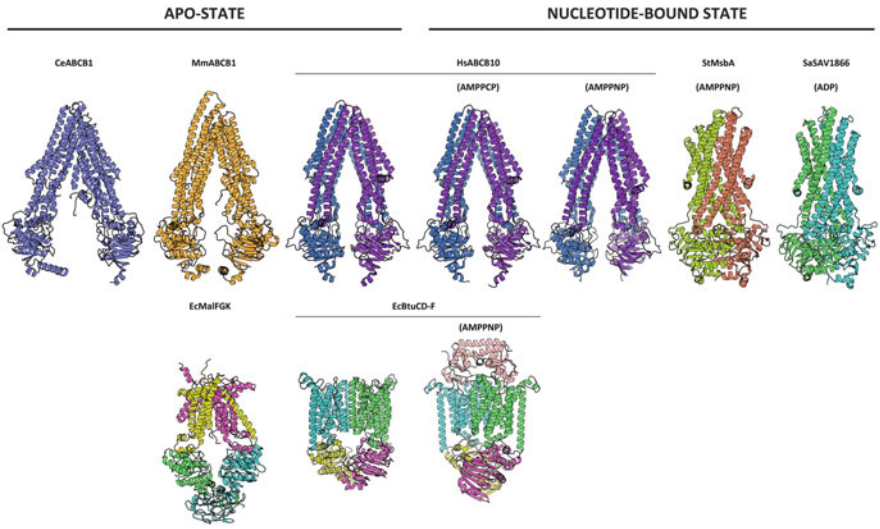

Fig. 5 Currently available crystal structures of full ABC-transporters representative of the different nucleotide-binding states and catalytic conformations. Dissimilar polypeptide chains are displayed in discrete colors. The differences in TMD organization and length between exporter (*top*) and importer (*bottom*) architectures are easily perceived. The identity of nucleotide analogs trapped into each protein crystal is given in brackets

remains enormous (in *Arabidopsis* about 20 out of 130 have been functionally analyzed) and suitable model systems, carefully designed experimental setups and long-term efforts are required to try elucidating the naturally transported ligand. Second, very similar orthologous transporters may achieve different functions in their respective organisms, as it was demonstrated for ABCB1 that functions as a multidrug exporter in mammals or in the export of the essential phytohormone, auxin, in plants. Third, the shape and dimensions of ABC translocation chambers are a challenge by themselves and cannot be seen as typical substrate-binding enzymatic pockets. Finally, the ABC-transporter structural design is certainly very dynamic and spatial residue distribution within the cavity at different time points of the catalytic cycle may represent very dissimilar landscapes over short time lapses. However, these issues and the knowledge gathered so far allow hypotheses to be drawn and partially tackled.

2.2.1 A Conserved Backbone That Supports Numerous Functions

Despite the remarkable conservation of NBD folds and the credible conservation of the energy-transmitting NBD–TMD interface, is there a population of conserved residues that maintained the common ABC transmembrane architecture over evolution times? A simple amino acid sequence alignment analysis including all ABCB proteins from six plant species mapped on an ABCB structural model tends to show

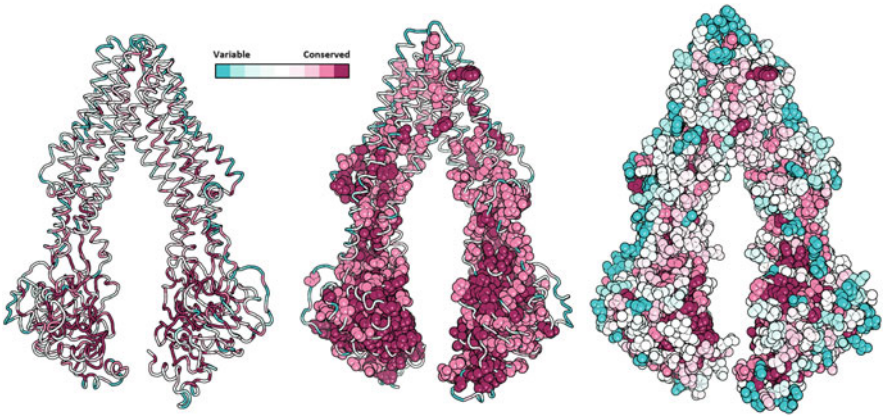

Fig. 6 Structural conservation in plant ABCB architectures. Color-coded ribbon (*left*) and sphere (*right*) representations of the conservation status of all ABCB proteins found in six separated plant species (*Arabidopsis tahliana, Populus trichocarpa, Brachypodium distachyon, Oryza sativa, Zea mays*, and *Vitis vininfera*). Full-length sequences were obtained from the Aramemnon database (aramemnon.botanik.uni-koeln.de) and conservation scores were calculated using the ConSurf server (http://consurf.tau.ac.il) and mapped on an inward-facing ABCB homology model. Note the apparent low conservation of surface residues compared to residues buried in the protein's core. Besides the preserved nucleotide-binding folds, sphere representation of the most conserved residues (*center*) reveals that both the NBD–TMD interface and α-helices essential rigid body constituents are shared between discrete ABCB transporters

that core hydrophobic residues buried within the membrane-spanning helices support a shared build among transporters with unrelated apparent functions (Fig. 6). In contrast, residues exposed to the intracellular medium display no evident conservation patterns (Fig. 7). Therefore these amino acids could be seen as the most promising candidates holding substrate selectivity. Careful analysis of conservation motifs in the translocation chamber of a given transporter with defined substrate specificity, assuming that its function has been maintained in evolutionary distant species, would be a good starting point to explore the structure to function relationship in solute transport. The coevolution of core residues building the ABC consensus architecture has been recently assessed in mammalian B and C ABC subfamilies (Gulyas-Kovacs 2012) with similar output: distinct amino acid pairs coevolved to maintain rigid body-like constituents of the transporters structure and preserve key elements assuring conformational reshuffling. This simple line of work could be extended to larger or narrower phylogenetic groups and help understanding the gain in ABC selectivity under evolution pressure.

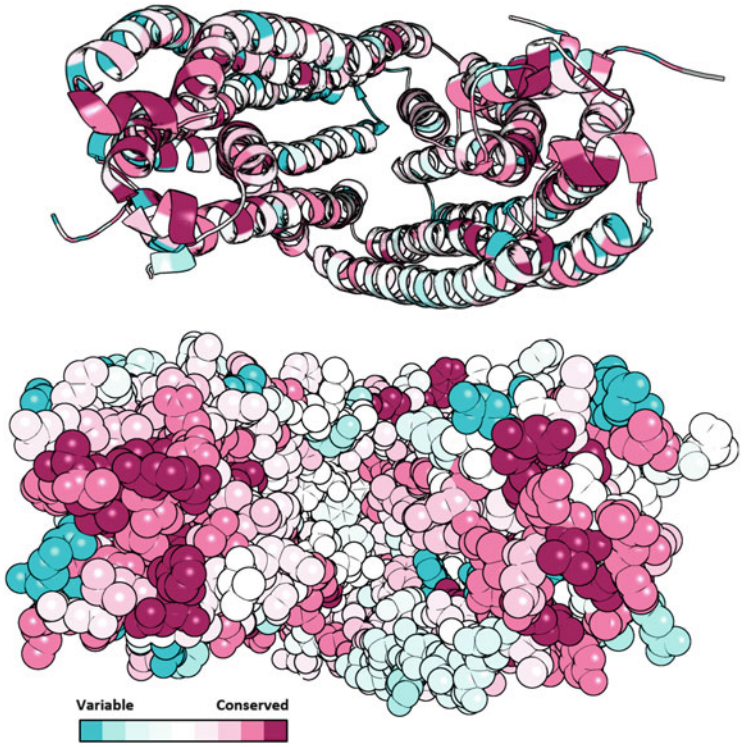

Fig. 7 Cytosolic view of the inward-facing TMD translocation chamber and its residue conservation according to data presented in Fig. 6. *Top*, cartoon representation helps in appreciating the backbone conservation status; NBDs have been omitted for clarity. *Bottom*, spherical surface representation of surface residues facing the cytosolic medium. Note that the proximal, highly conserved residues at the entry of the chamber define the coupling helices

2.2.2 The Substrate Recognition Competence Lies in a Limited Number of Chamber Surface Residues

A differential approach using mammalian and plant ABCB1 (or the close plant homolog ABCB19; Geisler and Murphy 2006) sequences and molecular models has been previously attempted (Bailly et al. 2012). From the high sequence conservation status of both human and *Arabidopsis* orthologs (≈40 % identity, ≈70 % homology), the authors postulated that the observed different recognition competence toward substrates, bulky hydrophobic, and small organic acidic compounds, respectively, could be hold by residues exposed to the transmembrane cavity. Therefore, sequence analysis, robust comparative homology modeling of a variety of plant and mammalian ABCB1 proteins, and exhaustive in silico docking simulations were performed to deduce kingdom-specific substrate-binding domains. On one hand, mammalian ABCB1 sequences displayed a strong and large spatial

conservation of chamber hydrophobic residues in line with the polyspecific substrate recognition proposed for *Hs*ABCB1 (Aller et al. 2009; Klepsch et al. 2011), thus suggesting the recent evolution of multidrug export in mammalian proteins (Fisher et al. 2012). On the other hand, the auxin transport function of plant ABCB1/19 transporters has been inherited from primary land plant species, inferring the early specialization of plant ABCB auxin exporters (Knoller et al. 2010). In this study, intrakingdom conserved polar residues, not shared between plant and animal exporters, appeared to correlate well with plant ABCB preferential sites in drug-binding computer simulations. Moreover, these putative ligand-binding regions suggested a rationale for the observed solute-selectivity in transport experiments (Bailly et al. 2012). Computation of surface electrostatics revealed a pronounced level of divergence in electrostatic properties correlating with the discrete substrate specificities of the transporters as reported earlier for different ABCB subfamily members (Flanagan and Huber 2007). This network of electrostatic interactions at the walls of the translocation chamber is anticipated to provide a basis for the substrate specificity in this region. Analogous efforts have been performed with human ABCB1 to reconcile structural information, in silico predictions, and biochemical evidence (Aller et al. 2009; Klepsch et al. 2011; Bikadi et al. 2011); nevertheless, despite the convincing methodology of comparative metadata analysis, the information collected in such studies await experimental validation.

2.2.3 A Very Large Chamber for Rather Small Ligands

The size of the translocation chamber can be addressed in a different angle, independently from the actual functional volume prevailing in vivo. Indeed, so far only hydrophobic substrates bound within the region of the translocation chamber aligned with the outer lipid layer of the membrane were recorded in crystallographic data. As depicted in Fig. 4, ABCB inward-facing conformers do not grant access from the outer membrane leaflet or the outer medium to the substrate. However, lipids and at least partially hydrophobic substrates would be allowed to cross loosely packed transmembrane helices and enter putative substrate-binding sites from the inner leaflet (Aller et al. 2009). In the published mouse ABCB1 structure, drugs with these properties (the cyclic peptides QZ59-RRR and QZ59-SSS; Aller et al. 2009; Li et al. 2014) were snapshot at the very top of the transport cavity, therefore virtually nested around residues in plane with the outer membrane leaflet. Remarkably, these residues overlap with other biochemically determined drug-binding sites such as for verapamil in human ABCB1 (Li et al. 2014; Loo and Clarke 2001). This transport route is consistent with older data suggesting that ABC-type pumps can function as hydrophobic *vacuum cleaners*, directly clearing lipid-partitioned substrates out of the membrane (Higgins and Gottesman 1992; Sharom et al. 2005). While this model fits well the multidrug exporters' features, it requires more efforts to be adapted to transporters extruding hydrophilic drugs. However, taking the plant ABCB1 as an illustration,

this general feature cannot be currently excluded and a certain logic in the distribution of solutes can be imagined. The pK_a (4.75) of the prominent auxin indole-3-acetic acid (IAA) drives the hormone from the acidic apoplast into the plant cell cytoplasm via lipophilic diffusion and is thought to be mainly trapped in its anionic state in this neutral compartment (Peer et al. 2011). In silico docking simulations nested IAA-binding in regions of the translocation cavity analogous to murine ABCB1 experimental substrate-binding regions (Bailly et al. 2012), therefore, in a scenario where the auxin anion is not the actual substrate and would not enter the cytosolic face of the transporter, a portion of the protonated form of the hormone transiting through the membrane bilayer could become available to entry sites in the membrane-spanning regions. It is yet too early to advocate for a unique, membrane-mediated mode of delivery of substrates into eukaryotic ABC cavities and the diversity of transported solutes known so far is a challenging pitfall for prediction software or mutagenesis designs. The possibility remains that each ABC solute type displays an exclusive entry path, but the structural and functional conservation within the transporters' family over evolution times does not sustain this idea.

2.2.4 A Hollow Ligand-Binding Site

The apparent structural reshuffling of the TMDs suggested by the available crystallographic data and supported by biochemical evidence (Moradi and Tajkhorshid 2013; Wise 2012) implies that ligand-binding sites in the inward- and outward-facing conformations of the transporters may not be equivalent. Residues defining a binding site in one initial conformer may be sufficiently modified in the final conformation to not accept the substrate anymore. Figure 8 depicts the transmembrane arrangements and the putative preferential binding site for auxin in plant ABCB1 proteins in both states. Key residues participating in this binding pocket in the inward-facing conformer are displaced away from the substrate-accepting cove in the outward-facing conformer and eventually face the outer medium, get hidden in the TMD hydrophobic core or buried into the lipid bilayer. Consequently, the binding site vanishes and putative protein–ligand interactions are expected to be disrupted. The TMD movements initiated by the coupling of substrate and nucleotide binding are therefore directly altering the transporter molecular affinity for its ligand, thus achieving a cycle of sequential specific recognition and release of the transported ligand. This mechanism elegantly describes the unidirectional transport of the solute across the membrane barrier.

3 A Well-Accepted but Highly Debated Mechanism

ABC-proteins functioning as pumps require at least three distinct structural features that have been observed in crystallographic data. First, the transporter should contain an opening big enough to admit the translocated substrate and that

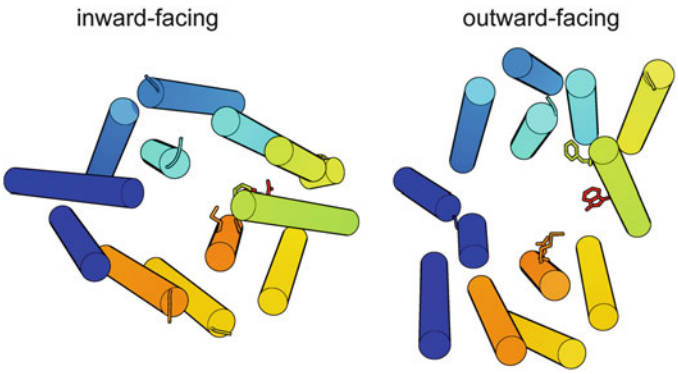

Fig. 8 The TMD reorganization induces changes in substrate affinities for each conformer. External view of the *A. thaliana* ABCB1 transmembrane domain inward- (*left*) and outward-facing (*right*) conformation (Bailly et al. 2012). The three residues represented as sticks protruding from two transmembrane helices (*cylinders*) describe a putative inward-facing pocket for auxin (*red sticks*). This site coordination fades away in the outward-facing conformer along with the large transmembrane helices movements, thus characterizing the substrate extrusion step

segregates it from the medium. Second, the protein should adopt a minimum of two conformations to sequentially expose its opening to both sides of a given membrane. Finally substrate-binding sites within the opening should display different affinities towards the substrate in the two conformations (Jardetzky 1966; Moradi and Tajkhorshid 2013; Hollenstein et al. 2007). Regardless of the structural discrepancies observed in the transmembrane folds of ABC importers and exporters, this elementary mechanism can be extended to virtually all ABC-transporters and differences in transport specificity would only call for an adaptation of binding sites within the translocation chamber.

The current views of ABC-transport mechanism (Hollenstein et al. 2007; Holland 2011; Jones and George 2013) start with substrate entry into the proteins' cavity open to the delivery side (Doshi and van Veen 2013). This cavity is sealed to the other side of the lipid bilayer and so does not represent a pore but can rather be seen as a double-door entrance. Therefore transport of the substrate across the membrane implicates structural rearrangements of the TMDs that lead to closing at the delivery side and subsequent or coordinated opening at the opposite side of the bilayer. This motion is supported by the ATP-driven closure of the NBD dimer interface that brings the portion of the TMDs that formed the entry door close together in a movement that has been compared to forceps levers (Chen et al. 2003). The allosteric transition needed to perform such rearrangements of transmembrane helices has been suggested to be mediated by the ICL regions of the TMDs that do not partition into the lipid bilayer but extend into the medium and directly interact with NBDs.

In detail, these polar segments, which are significantly longer in exporters, generally consist of a long–short–long α-helices sequence that loops between transmembrane segments into the inner compartment. The short helix, also

known as coupling helix (CHX), directly penetrates into the cove formed by the two NBD subdomains, thus interacting with segments critical to ATP hydrolysis such as the Q-loop (Dawson and Locher 2006; Hollenstein et al. 2007). Therefore the ICLs largely constitute the TMD–NBD interfacial region that transduces the structural changes between the two observed conformations in response to ATP binding/hydrolysis in the NBDs and substrate binding in the TMDs.

Whereas ABC-importers possess per module only one *bona fide* coupling helix that contacts the contiguous NBD, exporters architectures exhibit a second ICL coupling helix in each TMD that exclusively cross-contacts with the NBD from the opposite monomer (Dawson and Locher 2006; Loo et al. 2013). This domain swapping therefore maintains the two protomers in solid imbrications where the soluble extension of the sixth transmembrane helix and the first ICL from one protomer combine with the second ICL from the opposite protomer to create a stable globular domain principally associated with the NBD following the sixth transmembrane helix. Moreover, transmembrane helices four and five contact helices from the opposite protomer, shaping the translocation chamber in a distinctive V closed to the outer space. Following nucleotide binding and NBD dimer formation, the ICL domains get closer thus shutting the former inner entry side and switch the TMD conformation to the inverse V shape exposing the newly formed cavity to the outer. After extrusion of the solute, ATP-hydrolysis and dissociation of the NBD dimer weakens the hydrogen and salt-bridge interactions between the X-loops of NBDs and the ICL bundles of TMDs leading to a reopening of the transporter cavity to the inner side and closure to the outer (Loo et al. 2005; Sharom et al. 2005; Higgins and Gottesman 1992). This event resets the transport cycle to its initial substrate- and nucleotide-accepting state, so one can imagine that a vectorial, dynamic pathway for the substrate is selectively maintained during the transport sequence.

Although molecular dynamics simulations have been performed to evaluate the transition switch between the inward- and outward-facing conformations in exporter structures (Moradi and Tajkhorshid 2013; Weng et al. 2012; Ma and Biggin 2013), the trajectories of allosteric changes occurring in the opening and closing of the TMD and the exact coupling to NBD movements remain elusive. In the export mechanism suggested by the Sav1866 and MsbA nucleotide-bound structures, subtle translational shifts of NBD monomers along the interface of the NBD dimer are combined to a movement that twists the TMD modules from one conformation to the other (Hollenstein et al. 2007; Dawson and Locher 2007). Recent data from ABCB structures obtained in the nucleotide-free state revealed large gaps between the NBDs (Shintre et al. 2013; Jin et al. 2012; Aller et al. 2009), thus supporting a model with broad NBD movements and drastic TMD rearrangements during the catalytic cycle. Moreover, the recently revised mouse ABCB1 structure showed no major conformational difference with or without bound substrate (Aller et al. 2009), inferring that substrate-binding energies are not sufficient to promote TMD reorganizations and questioning how substrate recognition would promote ATP binding at all. However the same gapped conformations were observed for human ABCB10 structures in nucleotide-bound states

(Shintre et al. 2013). Therefore the proposed mechanism in which ATP binding provides the free energy to tightly close the NBD dimer and perform the power stroke of the transport cycle has to be carefully assessed and has possibly been biased by the use of conformation-trapping ATP analogs. The observed distances and orientations between NBD monomers in apo-state structures seem incompatible with a closure solely generated by electrostatic forces changes driven by nucleotide binding or hydrolysis. Certain sets of biochemical evidence, including cysteine cross-linking experiments in human ABCB1 (Zolnerciks et al. 2007; Doshi et al. 2010), vanadate trapping (Qu et al. 2003), and solvent accessibility assessments (Grote et al. 2008) tended to demonstrate that NBDs do not fully dissociate during the catalytic cycle. Yet they contradict the observed nucleotide-free structures of ABC-exporters. Additional evidence, ideally coordinates representing different transition steps in the catalytic cycle of the same transporter, are needed to fully elucidate the detailed functioning of these very ancient machineries. One certainty is that the strong structural and functional homology found in all ABC-transporters at and surrounding the ATP-hydrolytic site argues for a common principle where crosstalks between the NBD and TMD modules transmit the information through the ICL interface, with a probable direct read or control of the nucleotide-binding site status by coupling helices like it was proposed by molecular dynamics simulations (Ma and Biggin 2013; Moradi and Tajkhorshid 2013; Weng et al. 2012).

4 ABC-Pump: A Sensitive Machine?

The ancient origin of ABC-transporters and their broad range of functions allow us to speculate on the multiple layers of control applied on such fundamental machinery for life forms. Beside transcriptional, translational, and posttranslational regulations controlling ABC activity (discussed in Chap. 13), physical parameters directly linked to the membrane targeting of these proteins may greatly influence their activity. The inconsistency of NBD positions in the apo-state and nucleotide-bound ABC conformers questions the solidity of protein coordinates obtained in the rigid and densely packed crystal environment when compared to the *in situ* fluid and dynamic behavior of a living membrane. It is known from early experimental work on mammalian transporters that the cellular membrane properties can greatly affect the kinetics of the ABC-proteins (dos Santos et al. 2007; Beck et al. 2013; Aanismaa and Seelig 2007). Factors influencing membrane fluidity such as lipid content (Fenyvesi et al. 2008), cytoskeleton components (Bacso et al. 2004), or integral/interacting proteins may thus impact the packing and rearrangements of the transporter TMDs during the transport-cycle (Aanismaa et al. 2008) and could participate in the functional integrity of the protein in its natural context. Furthermore a model in which phospholipids and membrane sterols directly participate in the binding of ABC-exporters substrates has been proposed (Kimura et al. 2007). This "cholesterol fill-in" model suggested that ABC proteins transporting either hydrophobic or

hydrophilic molecules may share analogous substrate binding sites that concomitantly recognize lipids or sterols. Thus these amphiphilic components could fill the empty space in the translocation chamber and help accommodate small substrates in the binding site, independently of their size or polarity. This conception is supported by the positive modulation of *Arabidopsis* ABCB19 activity and membrane stability by structural sterols (Titapiwatanakun and Murphy 2009; Rojas-Pierce et al. 2007). Since several ABC-transporters are known to translocate membrane constituents (Tarling 2013; King and Sharom 2012), the hypothesis in which substrates partitioning in the lipid bilayer could follow the lipid transport is not irrational in a thermodynamic point of view. Such a mechanism would provide an explanation for the observed differences in substrate preferences exerted by very close transporters and would partially agree with the sensitivity of ABCB activity to membrane cholesterol contents (Fenyvesi et al. 2008). It is well accepted that the size of the ABC translocation chamber would allow for multiple substrates to be simultaneously transported during the catalytic cycle and this concept was retained in the case of plant ABCB1/19 (Bailly et al. 2012). If this multiple small substrates-binding step would actually occur in a definite region of the transporter cavity analogous to large substrates binding sites, one could suggest that this event could mimic larger drugs recognition and impact more drastically the electrostatic environment than single compound binding. However, no crystallographic data yet supported this concept.

Finally, even though the amount and quality of data collected on ABC superstructures, one puzzling question remains unanswered: what would be the function of the so called linker domain in mono-peptidic ABC transporters? This stretch bridging the first NBD module to the second TMD module has no equivalency in importer structures and is absent in close prokaryotic dimers. However, its rather short size, enrichment in phosphorylation motifs, and good conservation among homologs and orthologs suggest that it does not represent a loose segment devoid of function. Cleavage of the human ABCB1 linker did not alter its overall structure or nucleotide hydrolysis activity but impacted ligand specificity (Sato et al. 2009). It was further demonstrated that linker phosphorylation promoted efflux activity in *Arabidopsis* ABCB1, while its removal blocked transport entirely (Henrichs et al. 2012). Hence, it was proposed that the linker region guarantees the tight coupling between ATP binding/hydrolysis and substrate recognition, presumably by interfering with the conformational changes of the catalytic cycle (Henrichs et al. 2012; Sato et al. 2009). Intriguingly, the spatial configuration of this segment was never trapped into protein crystals and de novo modeling of the linker domain using different algorithms often predicts a short connection path from the first NBD to the second TMD through the region of the cavity more distal to the membrane. Although it would be extremely surprising that the linker domain participates in the binding of substrates, it is obvious that it represents another layer of control of ABC function and selectivity in higher organisms.

5 Outlook

The large number of plant ABC-transporters and their frequent functional redundancy represent an exceptional opportunity to gain more insights in the elegant mechanism of these ancestral pumps. Plant species are an ideal source of material for acquiring genomic data and biochemical evidence, for they are a mature, fast-generated system for mutagenesis, gene expression, and propose robust methods to study solute transport at the organ, cellular, and subcellular levels. Lastly, plant species are probably the best examples of Life's extreme potential to adapt to its environment at the lowest cost; a potential reflected in the conservation of a core ABC-transport structure adjusted to multiple molecular functions.

References

Aanismaa P, Seelig A (2007) P-Glycoprotein kinetics measured in plasma membrane vesicles and living cells. Biochemistry 46:3394–3404

Aanismaa P, Gatlik-Landwojtowicz E, Seelig A (2008) P-glycoprotein senses its substrates and the lateral membrane packing density: consequences for the catalytic cycle. Biochemistry 47:10197–10207

Aller SG, Yu J, Ward A, Weng Y, Chittaboina S, Zhuo R, Harrell PM, Trinh YT, Zhang Q, Urbatsch IL, Chang G (2009) Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding. Science 323:1718–1722

Bacso Z, Nagy H, Goda K, Bene L, Fenyvesi F, Matko J, SZABO G (2004) Raft and cytoskeleton associations of an ABC transporter: P-glycoprotein. Cytometry A 61:105–116

Bailly A, Yang H, Martinoia E, Geisler M, Murphy AS (2012) Plant lessons: exploring ABCB functionality through structural modeling. Front Plant Sci 2:108. doi:10.3389/fpls.2011.00108

Beck A, Aanismaa P, Li-Blatter X, Dawson R, Locher K, Seelig A (2013) Sav 1866 from *Staphylococcus aureus* and P-Glycoprotein: similarities and differences in ATPase activity assessed with detergents as allocrites. Biochemistry 52:3297–3309

Bikadi Z, Hazai I, Malik D, Jemnitz K, Veres Z, Hari P, Ni Z, Loo TW, Clarke DM, Hazai E, Mao Q (2011) Predicting P-glycoprotein-mediated drug transport based on support vector machine and three-dimensional crystal structure of P-glycoprotein. PLoS One 6:e25815

Bouchard R, Bailly A, Blakeslee JJ, Oehring SC, Vincenzetti V, Lee OR, Paponov I, Palme K, Mancuso S, Murphy AS, Schulz B, Geisler M (2006) Immunophilin-like TWISTED DWARF1 modulates auxin efflux activities of Arabidopsis P-glycoproteins. J Biol Chem 281:30603–30612

Chen J, Lu G, Lin J, Davidson AL, Quiocho FA (2003) A tweezers-like motion of the ATP-binding cassette dimer in an ABC transport cycle. Mol Cell 12:651–661

Dawson RJ, Locher KP (2006) Structure of a bacterial multidrug ABC transporter. Nature 443:180–185

Dawson RJ, Locher KP (2007) Structure of the multidrug ABC transporter Sav 1866 from Staphylococcus aureus in complex with AMP-PNP. FEBS Lett 581:935–938

Dean M, Annilo T (2005) Evolution of the ATP-binding cassette (ABC) transporter superfamily in vertebrates. Annu Rev Genomics Hum Genet 6:123–142

dos Santos SM, Weber CC, Franke C, Muller WE, Eckert GP (2007) Cholesterol: coupling between membrane microenvironment and ABC transporter activity. Biochem Biophys Res Commun 354:216–221

Doshi R, van Veen HW (2013) Substrate binding stabilizes a pre-translocation intermediate in the ATP-binding cassette transport protein MsbA. J Biol Chem 288:21638–21647
Doshi R, Woebking B, van Veen HW (2010) Dissection of the conformational cycle of the multidrug/lipidA ABC exporter MsbA. Proteins 78:2867–2872
Fenyvesi F, Fenyvesi E, Szente L, Goda K, Bacso Z, Bacskay I, Varadi J, Kiss T, Molnar E, Janaky T, Szabo G, Vecsernyes M (2008) P-glycoprotein inhibition by membrane cholesterol modulation. Eur J Pharm Sci 34:236–242
Fisher C, Coleman T, Plant N (2012) Probabilistic orthology analysis of the ATP-binding cassette transporters: implications for the development of multiple drug resistance phenotype. Drug Metab Dispos 40:1397–1402
Flanagan JU, Huber T (2007) Structural evolution of the ABC transporter subfamily B. Evol Bioinform 3:309–316
Geisler M, Murphy AS (2006) The ABC of auxin transport: the role of p-glycoproteins in plant development. FEBS Lett 580:1094–1102
Geisler M, Blakeslee JJ, Bouchard R, LEE OR, Vincenzetti V, Bandyopadhyay A, Titpiwatanakun B, Peer WA, Bailly A, Richards EL, Ejendal KF, Smith AP, Baroux C, Grossniklaus U, Muller A, Hrycyna CA, Dudler R, Murphy AS, Martinoia M (2005) Cellular efflux of auxin catalyzed by the Arabidopsis MDR/PGP transporter AtPGP1. Plant J 44:179–194
George AM, Jones PM (2012) Perspectives on the structure-function of ABC transporters: the switch and constant contact models. Prog Biophys Mol Biol 109:95–107
Grote M, Bordignon E, Polyhach Y, Jeschke G, Steinhoff HJ, Schneider E (2008) A comparative electron paramagnetic resonance study of the nucleotide-binding domains' catalytic cycle in the assembled maltose ATP-binding cassette importer. Biophys J 95:2924–2938
Gulyas-Kovacs A (2012) Integrated analysis of residue coevolution and protein structure in ABC transporters. PLoS One 7:e36546
Gutmann DA, Ward A, Urbatsch IL, Chang G, van Veen HW (2010) Understanding polyspecificity of multidrug ABC transporters: closing in on the gaps in ABCB1. Trends Biochem Sci 35:36–42
Henrichs S, Wang B, Fukao Y, Zhu J, Charrier L, Bailly A, Oehring SC, Linnert M, Weiwad M, Endler A, Nanni P, Pollmann S, Mancuso S, Schulz A, Geisler M (2012) Regulation of ABCB1/PGP1-catalysed auxin transport by linker phosphorylation. EMBO J 31:2965–2980
Higgins CF (1992) ABC transporters: from microorganisms to man. Annu Rev Cell Biol 8:67–113
Higgins CF, Gottesman MM (1992) Is the multidrug transporter a flippase? Trends Biochem Sci 17:18–21
Higgins CF, Linton KJ (2004) The ATP switch model for ABC transporters. Nat Struct Mol Biol 11:918–926
Holland IB (2011) ABC transporters, mechanisms and biology: an overview. Essays Biochem 50:1–17
Holland IB, Blight MA (1999) ABC-ATPases, adaptable energy generators fuelling transmembrane movement of a variety of molecules in organisms from bacteria to humans. J Mol Biol 293:381–399
Hollenstein K, Dawson RJ, Locher KP (2007) Structure and mechanism of ABC transporter proteins. Curr Opin Struct Biol 17:412–418
Hrycyna CA, Gottesman MM (1998) Multidrug ABC transporters from bacteria to man: an emerging hypothesis for the universality of molecular mechanism and function. Drug Resist Updat 1:81–83
Isenbarger TA, Carr CE, Johnson SS, Finney M, Church GM, Gilbert W, Zuber MT, Ruvkun G (2008) The most conserved genome segments for life detection on earth and other planets. Orig Life Evol Biosph 38:517–533
Jardetzky O (1966) Simple allosteric model for membrane pumps. Nature 211:969–970
Jin MS, Oldham ML, Zhang Q, Chen J (2012) Crystal structure of the multidrug transporter P-glycoprotein from Caenorhabditis elegans. Nature 490:566–569

Jones PM, George AM (2013) Mechanism of the ABC transporter ATPase domains: catalytic models and the biochemical and biophysical record. Crit Rev Biochem Mol Biol 48:39–50
Jones PM, O'mara ML, George AM (2009) ABC transporters: a riddle wrapped in a mystery inside an enigma. Trends Biochem Sci 34:520–531
Kimura Y, Kodan A, Matsuo M, Ueda K (2007) Cholesterol fill-in model: mechanism for substrate recognition by ABC proteins. J Bioenerg Biomembr 39:447–452
King G, Sharom FJ (2012) Proteins that bind and move lipids: MsbA and NPC1. Crit Rev Biochem Mol Biol 47:75–95
Klein M, Burla B, Martinoia E (2006) The multidrug resistance-associated protein (MRP/ABCC) subfamily of ATP-binding cassette transporters in plants. FEBS Lett 580:1112–1122
Klepsch F, Chiba P, Ecker GF (2011) Exhaustive sampling of docking poses reveals binding hypotheses for propafenone type inhibitors of P-glycoprotein. PLoS Comput Biol 7:e1002036
Knoller AS, Blakeslee JJ, RIchards EL, Peer WA, Murphy AS (2010) Brachytic2/ZmABCB1 functions in IAA export from intercalary meristems. J Exp Bot 61:3689–3696
Kretzschmar T, Burla B, Lee Y, Martinoia E, Nagy R (2011) Functions of ABC transporters in plants. Essays Biochem 50:145–160
Kubes M, Yang H, Richter GL, Cheng Y, Mlodzinska E, Wang X, Blakeslee JJ, Carraro N, Petrasek J, Zazimalova E, Hoyerova K, Peer WA, Murphy AS (2011) The Arabidopsis concentration-dependent influx/efflux transporter ABCB4 regulates cellular auxin levels in the root epidermis. Plant J 69:640–654
Li J, Jaimes KF, Aller SG (2014) Refined structures of mouse P-glycoprotein. Protein Sci 23:1091–1098
Locher KP, Lee AT, Rees DC (2002) The *E. coli* BtuCD structure: a framework for ABC transporter architecture and mechanism. Science 296:1091–1098
Loo TW, Clarke DM (2001) Defining the drug-binding site in the human multidrug resistance P-glycoprotein using a methanethiosulfonate analog of verapamil, MTS-verapamil. J Biol Chem 276:14972–14979
Loo TW, Bartlett MC, Clarke DM (2005) ATP hydrolysis promotes interactions between the extracellular ends of transmembrane segments 1 and 11 of human multidrug resistance P-glycoprotein. Biochemistry 44:10250–10258
Loo TW, Bartlett MC, Clarke DM (2013) Human P-glycoprotein contains a greasy ball-and-socket joint at the second transmission interface. J Biol Chem 288:20326–20333
Ma J, Biggin PC (2013) Substrate versus inhibitor dynamics of P-glycoprotein. Proteins 81:1653–1668
Martinoia E, Klein M, Geisler M, Bovet L, Forestier C, Kolukisaoglu U, Muller-Rober B, Schulz B (2002) Multifunctionality of plant ABC transporters–more than just detoxifiers. Planta 214:345–355
Mason DL, Michaelis S (2002) Requirement of the N-terminal extension for vacuolar trafficking and transport activity of yeast Ycf1p, an ATP-binding cassette transporter. Mol Biol Cell 13:4443–4455
Moradi M, Tajkhorshid E (2013) Mechanistic picture for conformational transition of a membrane transporter at atomic resolution. Proc Natl Acad Sci U S A 110:18916–18921
Peer WA, Blakeslee JJ, Yang H, Murphy AS (2011) Seven things we think we know about auxin transport. Mol Plant 4:487–504
Procko E, O'mara ML, Bennett WF, Tieleman DP, Gaudet R (2009) The mechanism of ABC transporters: general lessons from structural and functional studies of an antigenic peptide transporter. FASEB J 23:1287–1302
Qu Q, Russell PL, Sharom FJ (2003) Stoichiometry and affinity of nucleotide binding to P-glycoprotein during the catalytic cycle. Biochemistry 42:1170–1177
Rojas-pierce M, Titapiwatanakun B, Sohn EJ, Fang F, Larive CK, Blakeslee J, Cheng Y, Cutler SR, Peer WA, Murphy AS, Raikhel NV (2007) Arabidopsis P-glycoprotein19 participates in the inhibition of gravitropism by gravacin. Chem Biol 14:1366–1376

Sato T, Kodan A, Kimura Y, Ueda K, Nakatsu T, Kato H (2009) Functional role of the linker region in purified human P-glycoprotein. FEBS J 276:3504–3516

Saurin W, Hofnung M, Dassa E (1999) Getting in or out: early segregation between importers and exporters in the evolution of ATP-binding cassette (ABC) transporters. J Mol Evol 48:22–41

Sharom FJ (2008) ABC multidrug transporters: structure, function and role in chemoresistance. Pharmacogenomics 9:105–127

Sharom FJ, Lugo MR, Eckford PD (2005) New insights into the drug binding, transport and lipid flippase activities of the p-glycoprotein multidrug transporter. J Bioenerg Biomembr 37:481–487

Shintre CA, Pike AC, Li Q, Kim JI, Barr AJ, Goubin S, Shrestha L, Yang J, Berridge G, Ross J, Stansfeld PJ, Sansom MS, Edwards AM, Bountra C, Marsden BD, von Delft F, Bullock AN, Gileadi O, Burgess-brown NA, Carpenter EP (2013) Structures of ABCB10, a human ATP-binding cassette transporter in apo- and nucleotide-bound states. Proc Natl Acad Sci U S A 110:9710–9715

Shitan N, Bazin I, Dan K, Obata K, Kigawa K, Ueda K, Sato F, Forestier C, Yazaki K (2003) Involvement of CjMDR1, a plant multidrug-resistance-type ATP-binding cassette protein, in alkaloid transport in *Coptis japonica*. Proc Natl Acad Sci U S A 100:751–756

Tarling EJ (2013) Expanding roles of ABCG1 and sterol transport. Curr Opin Lipidol 24:138–146

Titapiwatanakun B, Murphy AS (2009) Post-transcriptional regulation of auxin transport proteins: cellular trafficking, protein phosphorylation, protein maturation, ubiquitination, and membrane composition. J Exp Bot 60:1093–1107

Tusnady GE, Sarkadi B, Simon I, Varadi A (2006) Membrane topology of human ABC proteins. FEBS Lett 580:1017–1022

Verhalen B, Wilkens S (2011) P-glycoprotein retains drug-stimulated ATPase activity upon covalent linkage of the two nucleotide binding domains at their C-terminal ends. J Biol Chem 286:10476–10482

Verrier PJ, Bird D, Burla B, Dassa E, Forestier C, Geisler M, Klein M, Kolukisaoglu U, Lee Y, Martinoia E, Murphy A, Rea PA, Samuels L, Schulz B, Spalding EJ, Yazaki K, Theodoulou FL (2008) Plant ABC proteins–a unified nomenclature and updated inventory. Trends Plant Sci 13:151–159

Ward A, Reyes CL, Yu J, Roth CB, Chang G (2007) Flexibility in the ABC transporter MsbA: alternating access with a twist. Proc Natl Acad Sci 104:19005–19010

Weng J, Fan K, Wang W (2012) The conformational transition pathways of ATP-binding cassette transporter BtuCD revealed by targeted molecular dynamics simulation. PLoS One 7:e30465

Westlake CJ, Cole SP, Deeley RG (2005) Role of the NH2-terminal membrane spanning domain of multidrug resistance protein 1/ABCC1 in protein processing and trafficking. Mol Biol Cell 16:2483–2492

Wise JG (2012) Catalytic transitions in the human MDR1 P-glycoprotein drug binding sites. Biochemistry 51:5125–5141

Zolnerciks JK, Wooding C, Linton KJ (2007) Evidence for a Sav 1866-like architecture for the human multidrug transporter P-glycoprotein. Faseb J 21:3937–3948

It Takes More Than Two to Tango: Regulation of Plant ABC Transporters

Markus Geisler

Abstract ABC transporters require a tight regulation of their transport activity as they often distribute substrates that are either toxic or of metabolic or developmental function. Moreover in contrast to secondary active transporters or channels they represent in energetic terms rather inefficient ATP-consuming nano-machines.

The regulation of mammalian ABC transporters has been deeply investigated in the last three decades because malfunction of many ABC transporters is causing socially and economically relevant diseases. However, despite their striking over-representation and their emerging important developmental and physiological function, surprisingly few is so far known on the regulation of plant ABCs. Therefore, in this chapter we have compared our recent knowledge on plant and non-plant ABC transporters in respect to their post-transcriptional regulation. Despite the limited information on plant ABC regulation it becomes more and more clear that many—if not all—regulatory mechanisms found for mammalian ABC transporters are also found in plants. We highlight in more detail some interesting examples on Arabidopsis ABCB regulation where plant research provided a deep and integrated understanding of ABC regulation that might serve as a blueprint for clinical research.

1 Introduction

Most members of the ATP-binding cassette (ABC) transporter superfamily function as primary active (ATP-dependent) efflux pumps of a variety of substrates, including metabolic products, sugars, vitamins, metal ions, peptides, lipids, hormones,

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sterols, and various endogenous and xenobiotic drugs (Linton 2007). Functionality of ABC transporters is provided by a minimum of four domains to form a functional transporter: two transmembrane domains (TMDs), thought to define substrate specificity and two nucleotide-binding domains (NBDs) shown to hydrolyze ATP, finally releasing the bound substrate during the reaction cycle (Linton 2007; see also chapter "Monocot ABC Transporters").

For a minimum of two reasons, ABC transporters require a tight regulation of their transport activity: First, they distribute substrates that are partially either toxic or of metabolic or even developmental function. And second, in contrast to secondary active transporters or channels they represent in energetic terms rather inefficient ATP-consuming nano-machines, because of their rather inappropriate ATP/substrate ratio (Linton 2007). However, the regulation of mammalian ABC transporters has been deeply investigated in the last three decades due to other reasons: malfunction of many ABC transporters is causing socially and economically relevant diseases. For example, point mutations in the ABCC protein, Cystic Fibrosis Conductance Regulator (CFTR/ABCC7), functioning as an ion channel that transports chloride and thiocyanate ions across epithelial cell membranes, lead to cystic fibrosis (Cant et al. 2014). Other ABCC genes encode for sulfonylurea receptors (SUR), which are the molecular targets of the sulfonylurea class of antidiabetic drugs whose mechanism of action is to promote insulin release from pancreatic beta cells. The SUR1 protein encoded by the *ABCC8* gene is associated with congenital hyperinsulinism and susceptibility to type-2 diabetes (Seino et al. 2012). Another disease, X-linked adrenoleukodystrophy (X-ALD), is an inherited metabolic storage disorder characterised by impaired peroxisomal β-oxidation. The accumulation of very long chain fatty acids (VLCFAs) was found to be due to a mutation of the *ABCD1* gene, which encodes an ABC transporter known as adrenoleukodystrophy protein (ALDP; Morita and Imanaka 2012; see chapter "Plant Peroxisomal ABC Transporters: Flexible and Unusual" for details). Some mutated ABC transporters cause human disorders related to lipid transport and metabolism, including Tangier and Stargardt disease (Quazi and Molday 2011). Further many ABC transporters are indirectly responsible for a range of other inherited human diseases along with both bacterial and eukaryotic (including human) development of resistance to multiple drugs, causing pleiotropic (PDR) or multidrug resistance (MDR) phenomena. The most-studied ABC is probably human ABCB1/PGP1/MDR1 whose in vivo function and substrate is remarkably still unclear (Ambudkar et al. 2003; Modok et al. 2006; Calcagno et al. 2007; Sauna et al. 2007). However, upon its overexpression in cancer cells, ABCB1 (beside other ABCC-type transporters) is mainly responsible for its tragic role in exporting administered cytotoxic drugs, leading often to multi drug resistance (MDR) phenomena and finally to chemotherapeutic failure (Higgins 2007).

The superfamily of ABC transporters is highly expanded in the model plant Arabidopsis (Theodoulou 2000; Sanchez-Fernandez et al. 2001; Martinoia et al. 2002; Rea 2007; Verrier et al. 2008; 125 MB; >120 ABC transporters) and other plant species (Martinoia et al. 2002; Jasinski et al. 2003; Verrier et al. 2008) in comparison to multicellular organisms of comparable genome size. For example,

the genome of Drosophila (120 MB) encodes for 51 ABC transporters, *C. elegans* (97 MB) reveals 48, while the larger human genome (3.150 MB) contains only 47 ABC transporter genes (Verrier et al. 2008). Currently, gene duplication events (Jasinski et al. 2003), an evolutionary "need" due to the high number of plant secondary compounds (Martinoia et al. 2002), their sessile lifestyle and their ability for post-embryogenetic development (Kepinski and Leyser 2005) are being discussed. The latter is supported by the finding that plant members of different ABC transporter subclasses have been shown to be implicated directly or indirectly in the transport of different plant hormones over the plant plasma membrane: several members of the Arabidopsis ABCB and ABCG subfamilies, have been shown to be implicated in transport of the auxins, IAA (Noh et al. 2001; Geisler et al. 2005; Bouchard et al. 2006; Geisler and Murphy 2006; Bailly et al. 2008; Yang and Murphy 2009) and IBA (Ruzicka et al. 2010). Moreover, full and half-size members of the ABCG family were shown to transport the hormone abscisic acid (ABA; Kang et al. 2010; Kuromori et al. 2010; see chapter "ABA Transport by ABCG Transporter Proteins"), and finally, recently, a full-size PDR member of the Petunia ABCG family was suggested to transport the hormone strigolactone (Kretzschmar et al. 2012).

Mammalian and yeast ABC proteins have been investigated on multiple levels of transporter regulation including transcriptional (activators and repressors), post-transcriptional (splice variants), chromosomal (epigenetic modifications), translational (mRNA stability), and posttranslational (alteration of proteins) modifications. However, despite their over-representation and their important developmental and physiological function requiring an obvious tight regulatory control, surprisingly few is so far known on the regulation of plant ABCs, both on the posttranscriptional and on the transcriptional level. Therefore, in this chapter we will focus on the posttranscriptional regulation of plant ABC transporters in respect to their cellular trafficking, their proteolysis, their composition of surrounding membrane, their protein–protein interactions, their protein phosphorylation, and fine-regulation by modulatory drugs. We will compare the recent knowledge on plant and non-plant ABCs allowing us to judge if plants have acquired plant-specific regulatory pathways.

2 Cellular Trafficking

Like for all membrane transporters, the ER is the starting point of ABC proteins to enter the secretory pathway, which is best understood for human ABCB1 and ABCC7/CFTR. After correct folding in the ER, which is assisted by a range of folding factors and chaperones, including heat shock proteins, the ABCs move to the Golgi, where posttranscriptional modifications occur in the *trans*-Golgi network (TGN). The TGN is also the branching point between PM and endocytic pathway destinations. From there ABC transporter-containing vesicles are moved along the actin cytoskeleton directly to the PM or to endosomal pools (Kipp and Arias 2000).

ABC transporters undergo a dynamic endocytosis and cycling between an intracellular endosomal pool and the plasma membrane (Kipp and Arias 2000), which is again actin but not microtubule-dependent. Rab GTPases are involved in protein trafficking and recycling (Lam et al. 2012). Although, the exact role of Rab proteins in ABC trafficking and cycling is unclear, however, they most probably function in providing ABC polarity in polarized cells (see also chapter "Trafficking of ABCB-Type Auxin Transporters" for details).

The entire significance of extensive ABCs trafficking in endocytic and post-Golgi compartments may not yet be fully understood, however, it allows for posttranslational folding, maturation, quality control, and ER-associated degradation (ERAD). For example, FKBP38 was shown to be involved in maturation and enhancement of the steady-state level of CFTR and the voltage-dependent delayed rectifier potassium channel (HERG) that is responsible for the long QT syndrome (Walker et al. 2007). FKBP38 is a member of the FK506-binding proteins (FKBPs) that constitute a family of proteins that catalyze the *cis–trans* interconversion of peptide bonds preceding a proline residue. The prolyl isomerization is important for both de novo folding of nascent polypeptide chains and the regulation of activities of mature client proteins (Banasavadi-Siddegowda et al. 2011). FKBP52, a close FKBP homolog, is involved in the regulation of steroid receptor trafficking between the cytoplasm and the nucleus (Storer et al. 2011).

So far only the trafficking routes of ABCBs (ABCB1, ABCB4, and ABCB19) and ABCGs (ABCG37/PIS1) have been investigated in detail in plants. ABCB1, ABCB4, and ABCB19 localize predominantly symmetrically at the plasma membrane (PM) and require the activity of the FKBP42, TWISTED DWARF1 (TWD1), for PM presence and activity (Wu et al. 2010; Wang et al. 2013; see below). In addition, the secretory pathway of ABCB19 to the PM is regulated by GNL1 (Titapiwatanakun et al. 2009), a BFA-insensitive ARF-GEF that functions in COPI-coated vesicle formation and vesicle–cytoskeleton interactions in the secretory pathway (Geldner et al. 2004). Compared to the well-investigated and dynamic endosomal cycling of PIN proteins (Jurgens and Geldner 2007; Feraru and Friml 2008; Kleine-Vehn and Friml 2008), the trafficking routes of ABCBs have not yet been understood in detail but ABCBs seem to be less dynamic, which is mainly judged upon treatments with the fungal toxin brefeldin A (BFA), forcing PM proteins into endosomal aggregates. In contrast to ABCB1, that is rapidly found in endosomal compartments (BFA bodies; (Wang et al. 2013)), ABCB4 and ABCB19 associate strongly in the PM and show reduced recycling features between the PM and early endosomes in comparison to ABCB1 or also PIN1 and PIN2 (Titapiwatanakun et al. 2009). ABCB4 slowly endocytoses to the vacuole via Sorting Nexin1 (SNX1), and the pathway is GNOM independent but actin dependent (Cho and Cho 2012).

Like for mammalian ABCBs, also the actin cytoskeleton is providing the frame for vesicle secretion. ABCB19 localization is not strikingly affected by the actin inhibitor, Latrunculin B (Titapiwatanakun et al. 2009), but it affects ABCB4 endocytosis and targeting to the PM. However, intracellular aggregation of ABCB4 after treatment with Cytochalasin D is less sensitive than for PINs, which

might be due to a less dynamic ABCB4 trafficking (Cho and Cho 2012; for further details see chapter "Trafficking of ABCB-Type Auxin Transporters").

The finding that ABCB1, ABCB19, and ABCB4—but not PIN1 or PIN2—(Bouchard et al. 2006; Wu et al. 2010; Wang et al. 2013) are delocalized to the ER and widely degraded in *twd1* argues for a conserved function between FKBP38 and TWD1/FKBP42 in respect to PM secretion and/or protein stabilization. Interestingly, both FKBPs were found to be associated with the ER chaperone, HSP90 (Kamphausen et al. 2002; Geisler et al. 2003), and for both proteins a chaperone function has been demonstrated (Kamphausen et al. 2002; Banasavadi-Siddegowda et al. 2011). An Hsp70–Hsp90 chaperone system is well studied in the context of the conformational activation of a steroid receptor, which is required for its hormone binding (Czar et al. 1997). FKBP52, like FKBP38 and FKBP42, a tetratricopeptide repeat (TPR)-containing immunophilin, binds Hsp90 through its TPR domain. The fact that Hsp90 plays an important role in the maturation of both CFTR (Skach 2006) and the HERG channel (Peterson et al. 2012) and the association of plant and mammalian TWD1 orthologs with Hsp90, raises the possibility that a similar FKBP-Hsp90 chaperone system might facilitate conformational maturation of the interacting ABC transporters as well.

However, CFTR posttranslational folding but not CFTR synthesis was shown to be dependent on the FKBP38 PPIase activity (Banasavadi-Siddegowda et al. 2011). Moreover, the interaction of FKBP38 with Hsp90 through the TPR domain negatively impacts CFTR synthesis and its posttranslational folding. Further experiments will show if such a suggested pro-folding effect for FKBP38 in CFTR biogenesis mediated largely through its PPIase activity and regulated by Hsp90 holds also true for TWD1 (see Fig. 2).

The molecular mechanisms for regulatory ABCB modules in respect to protein–protein interaction/modulatory drugs and protein phosphorylation are summarized in more detail below.

Unlike IAA transporters of the PIN and ABCB families, ABCG37/PDR9/PIS1 and its homolog ABCG36/PDR8/PEN3 reside at lateral, outermost root plasma membrane domains where they act redundantly in export of the auxin and IAA precursor, IBA (Strader and Bartel 2009, 2011; Ruzicka et al. 2010). Delivery to this outer polar domain depends on ARF-GEF and actin function but does not require known molecular components of the apical or basal targeting. The outer polar delivery is, in contrast to known basal and apical cargo delivery mechanism in plants, mediated by polar secretion (Langowski et al. 2010). Interestingly, expression of ABCG36 (PEN3-GFP) in the root epidermis at the lateral, outward-facing domain matches perfectly that of TWD1 co-localized using anti-TWD1 (Bailly et al. 2013; Wang et al. 2013). Moreover, upon BFA treatment ABCG336 and TWD1 were found on the same endosomal compartment. If these findings simply reflect a lateral co-localization or functional interaction is unclear.

In summary, it appears that plant ABC trafficking and recycling, although less understood in comparison to their mammalian orthologs, employ overlapping pathways and components but are also partially distinct. This might be at least

partially connected to their reduced cellular polarity but also based on the fact that work on mammalian ABCs is mostly achieved using polarized cell lines.

3 Proteolysis

Misfolded human ABCB1 can be rapidly degraded and degradation of misfolded proteins is proteasome mediated (Loo and Clarke 1997), presumably by ER-associated protein degradation (ERAD). Mammalian ABCB substrates, such as cyclosporin A, that bind the drug-binding sites and facilitate conversion of misfolded intermediates into a more native conformation rescue misfolded ABCBs (Loo and Clarke 1997). As mentioned above, chaperones, such as FKBPs, have a significant impact on ERAD degradation of ABC transporters, shown for CFTR (Banasavadi-Siddegowda et al. 2011).

In this respect, the finding that Arabidopsis ABCBs are not only delocalized to the ER in *twd1* (Wu et al. 2010; Wang et al. 2013) but also heavily degraded (Wang et al. 2013) was highly interesting. As a result, the *twd1* mutant shows a strong reduction of polar auxin transport activity compared to the wild type (Geisler et al. 2003; Bailly et al. 2013). However, these findings imply an analogous, ERAD-like ABCB degradation that however awaits confirmation. This effect shown for B1, B4 and B19 seems to be specific as other PM proteins, including PIN1 and PIN2, were shown to be not affected (Bouchard et al. 2006; Wu et al. 2010; Wang et al. 2013). Proteolytic ABCB turnover may also play a role in the stabilization of PIN1 associated with ABCB19-containing membrane subdomains, as PIN1 is more resistant to degradation in those fractions (Titapiwatanakun and Murphy 2009).

4 Membrane Lipid Composition

It has been widely accepted that the plasma membrane is not a uniform phospholipid bilayer but instead is composed of distinct and specialized regions with varying proportions of lipids and proteins. Such small microdomains of 10–200 nm that are sphingolipid- and cholesterol-enriched are called “lipid rafts” (Laude and Prior 2004). Because lipid rafts are less fluid and more resistant to solubilization by nonionic detergents, such as Triton X-100, they are also referred to as detergent-resistant micelles (DRMs). A row of mammalian ABC transporters (including ABCB1/PGP1, ABCG5, ABCC2/MRP2, ABCB2/MDR2, BCRP, ABCD1, ABCC1, and ABCG2) has been reported on those membrane microdomains or to colocalize with lipid-raft markers (Bacso et al. 2004; Li and Prinz 2004; Radeva et al. 2005; dos Santos et al. 2007; Ismair et al. 2009; Klappe et al. 2009; Yun et al. 2013).

The lipid environment was demonstrated to alter the activity of ABC transporters. Cholesterol is important for maintaining the order and packing of lipids and proteins in membrane rafts. Therefore, a common approach to studying membrane compartmentalization is to assess transporter function under cholesterol-depleted conditions. For example, reduced membrane cholesterol lowers BCRP (Storch et al. 2007) and ABCB1 (dos Santos et al. 2007) transporter activity in overexpressing cell lines. How cholesterol depletion influences transporter function is not clear, but may involve protein–protein interactions within the membrane, modulation of transporter transmembrane domains, disassembly of membrane rafts, or interference of transporter binding to lipophilic substrates (Storch et al. 2007).

As such the lipid composition of biological membranes is closely related to the function of mammalian ABCBs (Dos Santos et al. 2007). Therefore it was of interest that using proteomic approaches, ABCB (together with PIN) proteins have been found on detergent-resistant membrane (DRM) fractions from Arabidopsis that are enriched in sterols as well as glucosylceramide (Borner et al. 2005; Titapiwatanakun et al. 2009).

However, current work in this respect has mainly focused on Arabidopsis ABCB19 shown to function coordinately with PIN1 to drive long-distance transport of the phytohormone auxin from the shoot to the root apex. ABCB19 exhibits a predominantly apolar plasma membrane (PM) localization and stabilizes PIN1 when the two proteins co-occur (Titapiwatanakun and Murphy 2009). Biochemical evidence associated ABCB19 and PIN1 with sterol- and sphingolipid-enriched PM fractions and mutants deficient in structural sterols and sphingolipids exhibited *abcb19* mutant-like phenotypes. ABCB19 imaging provided evidence that sterols function in trafficking of ABCB19 from the *trans*-Golgi network to the PM (Yang et al. 2013). These concepts are indirectly supported by the finding that mutants lacking a functional STEROL METHYLTRANSFERASE 1 (SMT1), required for the first step of sterol biosynthesis, show a drastic phenotype including disturbed cell polarity, auxin distribution, embryo development, and incorrect PIN1 and PIN3 (but not AUX1) locations (Willemsen et al. 2003).

The importance of sterols in defining the proper membrane environment was also used to explain the difficulties to functionally express ABCB19 (or PIN1) in heterologous systems, such as in baker's yeast (Petrasek et al. 2006; Blakeslee et al. 2007). However, *Schizosaccharomyces pombe* was recently found to be suitable for the expression of ABCB19 as a fully active transporter (Titapiwatanakun et al. 2009; Titapiwatanakun and Murphy 2009; Yang and Murphy 2009). This is thought to be due to the presence of plant-like sterol-enriched microdomains in *S. pombe*, which are not found in *S. cerevisiae* or Xenopus oocytes.

Future studies into the functional significance of ABC transporter expression in membrane rafts should provide insight into the complex membrane organization and compartmentalization of the plasma membrane.

5 Glycosylation

Glycosylation is the posttranslational protein modification of covalent sugars to proteins. This is typically performed in the rough endoplasmic reticulum and the Golgi apparatus and can occur at the amide side chain of asparagine (*N*-linked glycosylation) and at the hydroxyl side chain (*O*-linked glycosylation). Block of glycosylation can induce improper protein folding, protein degradation, impaired cellular trafficking to the cell surface, and altered transporter activity. For example, apically expressed efflux transporters ABCC2/MRP2, ABCG2/BCRP and ABCB1/MDR1 (Sinko et al. 2003; Diop and Hrycyna 2005; Mohrmann et al. 2005) were shown to be regulated by glycosylation and cellular trafficking.

Compared to that, nothing is known about the impact of plant ABC transporter glycosylation, which needs obviously to be addressed.

6 Protein–Protein Interactions

Protein–protein interactions (PPIs) regulate virtually all cellular processes by promoting appropriate cellular localization, activity, or stability of regulatory partners. When it comes to regulation of transport processes, especially ATP-driven ones, PPIs are thought to provide a quick and economic mode of temporal and spatial control of transport.

In the last decade, whole genome protein interaction maps (interactomes) providing a broad-based look at the functional and regulatory associations within the cell have been launched and completed for some model organisms, including *S. cerevisiae, D. melanogaster, C. elegans* (Lievens et al. 2010). However in these studies, PPIs between membrane proteins have been found to be underrepresented, most probably due to their hydrophobicity usually causing trouble in GAL4-based yeast-two hybrid (Y2H) systems. Also in proteomics studies that are currently being used widely in order to analyze co-immunoprecipitations (co-IPs), the need of detergent solubilization obviously results in the destruction but also creation of PPIs.

Therefore, several groups in the transport and ABC transporter field have launched individual small-scale PPI projects that focused on a limited subset of (ABC) transporters (Paumi et al. 2007, 2009) or interacting signaling components (Lalonde et al. 2008, 2010). A pioneer work in the ABC field was provided by the groups of Susan Michaelis and Igor Stagljar who developed a modified version of the split-ubiquitin membrane yeast two-hybrid (MYTH) technology using genomically integrated "bait" constructs, hence the designation iMYTH. iMYTH in a library-screening format resulted in the identification of six potential interacting partners of the yeast ABC transporter ABCC, yeast cadmium factor (YCF1) (Paumi et al. 2007). In a follow-up paper, they provided the ABC interactome of yeast by analyzing the 19 of all 22 yeast ABC transporters predicted

to have cytosolic termini, a requirement for MYTH (Snider et al. 2013). An unexpected outcome was the large number of functionally diverse interactors suggesting that ABC transporters have a more general involvement in cellular processes than previously suspected. Further, one interesting observation in the interactome was the tendency of full-size ABC transporters to interact with one another and the association of several ABC transporters with two major yeast zinc transport proteins (for details, see below).

Investigations of clinically relevant ABC transporters have early on focused on their regulation because this knowledge might provide us once with an option to treat diseases caused with their malfunction. In contrast, work on plant ABC transporters has with a few exceptions centered mainly around their physiological characterizations in respect to their substrate specificity, which is most cases was already puzzling enough. In respect to PPIs, work on ABC transporters has been mainly limited to the interaction with the FKBP42, TWISTED DWARF1 (TWD1), and with permease-like members of the PIN-FORMED (PIN) family in respect to auxin transport.

6.1 ABC Transporter-FKBP Interactions

6.1.1 ABCB–FKPB Interactions

First evidence that FKBPs might be involved in ABCB regulation by means of PPI was provided by the Heitman lab showing that yeast FKBP12 regulates MDR-like ABC transporter, MDR3, in yeast (Hemenway and Heitman 1996). This overall concept is supported by several other studies: FKBP12 is a subunit and inhibits basal signaling of two intracellular calcium release channels, the inositol 1,4,5-trisphosphate and ryanodin receptor (Cameron et al. 1995; Timerman et al. 1995).

Several plant ABC transporters were isolated in a yeast two hybrid screen for putative interactors using the soluble portion of TWD1 as bait in order to understand the pleiotropic developmental growth phenotype of *twd1*, characterized by a dwarf plant size, reduced cell elongation, disoriented growth of all organs, and misshapen (twisted) epidermal cells (Geisler et al. 2003). The rational to do so was based on the fact that TWD1 owns no detectable PPIase activity using standard test substrates but contains three repetitions of a so-called tetratricopeptide repeat (TPR; Geisler et al. 2003). This qualified TWD1 as a multidomain (high-molecular weight) FKBP, containing typically up to three N-terminal putative FK506-binding domains (FKBDs), typically followed by a TPR domain and a calmodulin-binding domain (both known to mediate protein–protein interactions to heat-shock proteins and calmodulin, respectively (Geisler and Bailly 2008). Both calmodulin and HSP90 in vitro binding to TWD1 was demonstrated although the physiological relevance of these interactions is entirely unclear.

The two-hybrid screen for TWD1 interactors resulted, beside HSP90 in the identification of C-terminal nucleotide binding domains (NBDs) of ABCB1 and

members of the ABCC/MRP family (see below). TWD1–ABCB1 interaction was verified by using in vitro pull-down assays, NPA affinity chromatography, Co-IP (Geisler et al. 2003) and Bioluminescence Resonance Energy Transfer in yeast (BRET; Bailly et al. 2008; Wang et al. 2013). Mapping of interacting domains demonstrated unexpectedly that not the C-terminal TPR domain but the N-terminal putative PPIase domain (FKBD) provided this interaction (Geisler et al. 2003). Interaction was not affected by immunosuppressant drugs, suggesting, together with the absence of a detectable PPIase activity, an evolutionary shift of function toward protein–protein interaction (Geisler et al. 2003; Geisler and Bailly 2007). Both interaction and putative ABCB regulation are provided by the N-terminal FKBD, as the soluble FKBD upon coexpression (or ScFKBP12) in yeast can functionally replace the full-length TWD1 (Bailly et al. 2008).

Initially due to a non-detectable PPIase activity for TWD1 (Geisler et al. 2003), TWD1 was thought to stabilize ABCs functionally by interaction with their C-terminal NBDs (Geisler et al. 2003, 2004). A puzzling finding was however that coexpression of ABCB1 with TWD1 (but not with Arabidopsis FKBP12) in yeast reduced ABCB1 IAA export activity to vector control levels as shown by transport and growth assays (Bouchard et al. 2006). This was surprising as it is the opposite of what one would have expected from previous in planta data, where auxin transport in *twd1* was reduced to *abcb1 abcb19* level (Geisler et al. 2003) being in line with overlapping phenotypes (Bailly et al. 2008, 2013; Geisler et al. 2003) TWD1 has also a negative impact on ABCB1 activity when co-expressed in the heterologous plant system *N. benthamiana* (Henrichs et al. 2012). However, in mammalian HeLa cells, TWD1 has an activating effect on ABCB1, suggesting that a third factor might be absent in yeast and tobacco (Bouchard et al. 2006). An alternative explanation is that TWD1 might compete for ABCB1 activation by high levels of yeast (or tobacco) FKBP12, the first shown to activate ABCB1 and mouse ABCB3/MDR3 (Hemenway and Heitman 1996). Assuming higher affinity and/or abundance of heterologous FKBP12 compared to TWD1, this would result in a net reduction of ABCB1-mediated auxin transport (Bailly et al. 2008; Henrichs et al. 2012). This concept is supported by the recent finding that yeast FKBP12 is able to widely complement *twd1* (Henrichs et al. 2012). A third but not exclusive explanation was given by the finding that TWD1 serves as a platform for recruiting the AGC kinase, PINOID (PID), shown to phosphorylate ABCB in planta. Interestingly, phosphorylation sites and their impact on auxin efflux vary with the presence of TWD1, which again is dependent on the choice of expression systems (for details, see Fig. 1 below).

An unexpected finding in respect to the ABCB1–TWD1 interaction was that coexpression in tobacco and different heterologous expression systems altered the capacity of ABCB1 to export the native auxin, IAA, but not to the synthetic auxin, 1-NAA, or the diffusion control, benzoic acid (Bouchard et al. 2006; Henrichs et al. 2012; Bailly et al. 2013). The molecular reasons for this specificity filter-like action of TWD1 are entirely unclear but the data suggest that enhancing the ABCB-TWD1 ratio might lead to a gain of substrate specificity, the opposite what might take place during multidrug resistance-related phenomena.

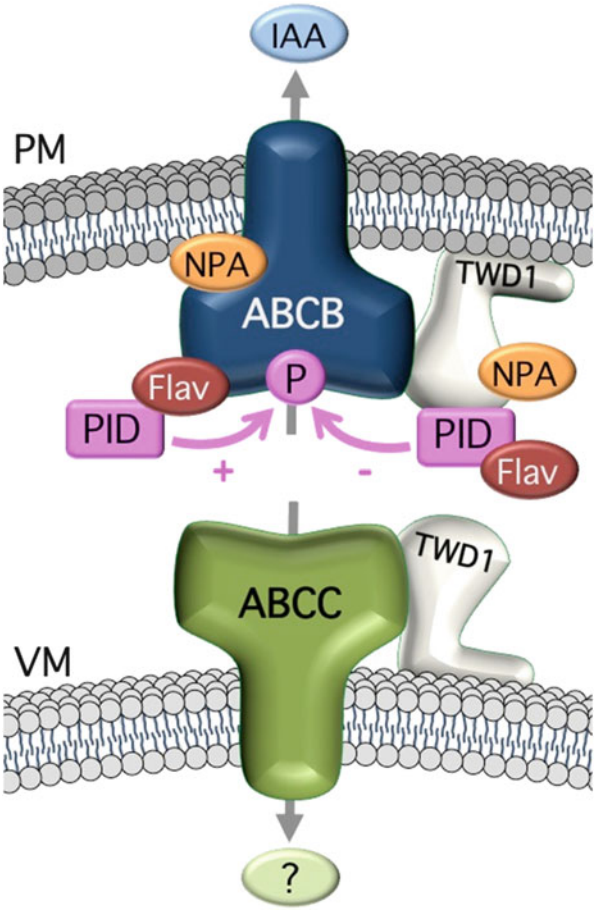

Fig. 1 Model featuring the interaction of TWD1/FKBP42 with plant ABCB and ABCC transporters. Proposed protein interactions between ABCB (*blue*) and ABCC (*green*) and the FKBP42, TWD1 (*grey*) on the plasma (PM) and vacuolar membrane (VM), respectively. Note that interactions occur between NBD2s of ABCBs (Geisler et al. 2003) and ABCCs (Geisler et al. 2004) but that interacting domains of TWD1 are FKBD and TPR domains, respectively. Interaction between ABCB1 and TWD1 is disrupted by NPA, thought to bind to the NBD–ICL interphase of ABCBs (Kim et al. 2010) or the FKBD of TWD1 (Bailly et al. 2008). This effect can be mimicked by binding of flavonols (Flav), such as quercetin, thought to bind to the NBDs of ABCBs (Peer et al. 2004). The AGC kinase PINOID (PID) was shown to phosphorylate ABCB1 at S634 of the linker resulting in inhibition of ABCB activity and NPA binding (−). In the presence of TWD1, ABCB1 phosphorylation by PID at an unknown residue results in ABCB1 activation (+) (Henrichs et al. 2012). Note, that flavonoids bind to ABCBs and PID (but not to TWD1) resulting in inhibition of transport by ABCB1 and ABCB1 phosphorylation, respectively

Employing different tools it appears that TWD1 in analogy to its mammalian ortholog, FKBP38 (Shirane and Nakayama 2003; Edlich and Lucke 2011) resides on multiple subcellular compartments, including the ER, the PM, and the vacuole

(Kamphausen et al. 2002; Geisler et al. 2003, 2014; Wu et al. 2010; Henrichs et al. 2012; Bailly et al. 2013; Wang et al. 2013). Interestingly, based on the finding that B1, B4- and B19-GFP are retained on the ER in *twd1*, a chaperone function independent of a PPIase activity for TWD1 in plasma membrane secretion of ABCBs was re-proposed (Wu et al. 2010). Later it was shown that ER retention was accompanied by protein degradation in *twd1* (Wang et al. 2013), resembling the overall role of FKBP38 in maturation and enhancement of CFTR steady-state levels (see above). These findings are in agreement with two alternative scenarios that are obviously not exclusive: absence of ABCB activity on the PM is caused either (1) by a lack of TWD1 chaperon function required for ABCB secretion from the ER to the PM, or, (2) by a block of ABCB activation/stabilization on the PM by a so far unclear mechanism. As a result, inactive ABCBs would be removed from the plasma membrane, and thus ER locations represented degradation locations.

6.1.2 ABCC–FKPB Interactions

As mentioned above, the ABCC protein, CFTR/ABCC7, is known to interact with a wide and growing array of protein partners in vivo that are critical for its channel activity (Monterisi et al. 2013). Such partner proteins include cytoskeletal proteins, other transporters and channels, receptors, kinases, and phosphatases (Li and Naren 2011). In the C-terminal region of CFTR, the interactions occur via a PDZ motif, providing direct interaction with at least six different PDZ scaffolding proteins.

In that respect it is important to recall that TWD1 has been shown to functionally interact with vacuolar ABCC1/MRP1 and ABCC2/MRP2 (Geisler et al. 2004). Importantly, using electron microscopy on HA-TWD1 plants, TWD1 has, beside its PM and ER locations, additionally been localized to the tonoplast (Kamphausen et al. 2002) and TWD1–ABCC1 interaction was verified by co-IP (Geisler et al. 2004). Unlike ABCB1, ABCC1 binds to the C-terminal tetratricopeptide repeat domain of TWD1, while domain mapping proved that TWD1 binds to a motif of AtMRP1 that resembles calmodulin-binding motifs. Interestingly, TWD1 was found to bind to isolated vacuoles and had a significant impact on the uptake of well-known substrates of vacuolar ABCCs, metolachlor-GS and estradiol-beta-glucuronide, as the in vivo substrates were not known at the time point of this study (Geisler et al. 2004). Currently they are seen as phytochelatin transporters providing resistance to arsenic, cadmium, and mercury (Park et al. 2012). However, the described transport activities might be evolutionary conserved, ABCC-intrinsic activities. Therefore, these transport capacities do not strictly exclude an involvement in vacuolar auxin transport, especially as the vacuole has recently been characterized as a further auxinic compartment (Ranocha et al. 2013).

The crystal structure of the TWD1 FKBD and the full-length protein without membrane anchor have been determined (Granzin et al. 2006; Weiergraber et al. 2006). Moreover, the NMR structure of the TWD1 FKBD has been released (Burgardt et al. 2012). Based on these, in silico-modeling of the protein–protein interaction with key interacting partners, ABCB1 and ABCC1, has facilitated the

prediction of docking sites at the molecular level. Although the docking domains of TWD1 that interact with the nucleotide-binding fold of ABCB- and ABCC-like ABC transporters are different (FKBD and TPR, respectively), both interactions involve overlapping surface areas on the transporters, suggesting a new paradigm for the regulation of ABC transporter activity (Granzin et al. 2006). Although it should be mentioned that this concept awaits experimental verification and harbors some technical problems: the MsbA structure used as a scaffold for NBD2 positioning had to be retracted due to software problems (Miller 2006). Moreover, docking distances were based on a C-terminal (class I) anchoring of TWD1 while newer data support a perpendicular, in-plane orientation of the membrane anchor (Scheidt et al. 2007; Bailly et al. 2013). However, our own work employing robust ABCB1 homology models (Bailly et al. 2011) that are based on the crystal structure of multidrug efflux pump Sav1866 (Dawson and Locher 2007) support a similar docking of the TWD1 FKBD domain to the NBD2 surface. Interestingly, the predicted ABCB1 docking surface is in direct vicinity to the A-loop, an essential mechanistic component of ABCs' canonical RecA-like ATPase subdomain and involved in ATP binding and hydrolysis, respectively.

Co-crystallization of TWD1 with interacting partners will be the method of choice to understand FKBP protein–protein interactions at the molecular level.

In summary, the above comparison argues for a conserved function between FKBP38 and TWD1/FKBP42 in respect to PM secretion and/or protein stabilization of ABCBs and possibly also on ABCCs, although the latter has not yet been demonstrated. However, the final mode of TWD1 chaperon action on ABCB secretion, stabilization, or activation as well as the contribution of a yet not demonstrated PPIase activity is still entirely unclear. For CFTR posttranslational folding but not CFTR synthesis the FKBP38 PPIase activity was shown to be essential (Banasavadi-Siddegowda et al. 2011). In any case, a comparison of *twd1* with *b1 b4 b19* plants might provide new insights. Moreover, recently also the closest homologue of ABCB4, ABCB21, was shown to function like B4 as facultative auxin im/exporter (Kamimoto et al. 2012). In that respect an analysis of TWD1–B4/B21 interaction is highly desirable. Another open question is the relevance of TWD1 membrane anchoring, which was suggested to increase the probability of contacts by reducing the spatiality of TWD1 diffusion thereby restraining the mobility of TWD1 and serving as a means to decouple the regulation of transporters located on different membranes (Scheidt et al. 2007). This was indirectly supported by the finding that expression of TWD1 lacking its IPM lead to enhanced ABCB-mediated auxin export resulting in elevated cell elongation and hypermorphism (Bailly et al. 2013).

6.2 ABC Transporter-Transporter Interactions

Different ABC transporters have been shown to function primarily as regulators of other transporters, including antiporters and channels. The-best investigated

example is probably the human sulfonylurea receptor (SUR/ABCC7) that regulates activity of the potassium ATP channel, KATP (Bessadok et al. 2011). KATP is particularly important in the regulation of insulin secretion from pancreatic cells and SUR is indispensable for normal channel function. SUR mutations thus increase the susceptibility to diabetes, myocardial infarction, and heart failure (Seino et al. 2012).

Recently, the functional association of several yeast plasma membrane ABCG transporters with two major yeast zinc transport proteins, ZRC1, a vacuolar H^+/zinc uptake antiporter, and ZRT1, a plasma membrane-localized, high- affinity zinc uptake transporter of unclear energization, was reported (Snider et al. 2013). Additionally iMYTH screens detected six new physical associations between ABCG transporters and confirmed three previously reported interactions.

Pharmacological evidence using sulfonylurea and potassium channel openers indicated the presence of CFTR- and SUR-type membrane proteins in the plasma membrane of plant guard cells (Leonhardt et al. 1999). Arabidopsis ABCC5/MRP5 was suggested to be directly involved in ion channel regulation and to represent a plant homologue of CFTR or SUR based on the findings that ABCC5 binds glibenclamide [66] and that stomata of *abcc5* mutants are insensitive to glibenclamide (Klein et al. 2003). In tobacco guard cell protoplasts ectopically expressing AtMRP5, the activity of the slow (S-type) anion channel was consistently reduced. In summary these results indicated that a plant ABC transporter can directly regulate ion channels most probably at the level of the S-type anion channel.

Previous work established that Arabidopsis ABCB and permease-like PIN proteins are able to function as independent auxin export catalysts (Petrasek et al. 2006). However, subcellular co-localization, yeast-two hybrid interaction and co-immunoprecipitation analyses provided clear evidence for distinct ABCB1,19-PIN1,2 pairings (Blakeslee et al. 2007; Rojas-Pierce et al. 2007; Kim et al. 2010). As was the case for ABCB/TWD1 pairs, interaction of ABCBs and PINs employ the C-terminal NBDs of the ABCBs binding the central cytoplasmic PIN loops (Blakeslee et al. 2007).

Co-expression of ABCBs in HeLa cells with PIN1 increased export, NPA-sensitivity and substrate specificity of B1/B19, while PIN2 had only significant effects on ABCB specificity (Blakeslee et al. 2007). Based on interaction and transport studies, there was no indication for functional ABCB interaction with putative H+/IAA antiporter, AUX1 (Blakeslee et al. 2007). However, functional co-expression of PIN1 reversed the import direction of ABCB4 in HeLa cells, while PIN2 enhanced ABCB4 activity (Blakeslee et al. 2007). However, trials to verify PIN–ABCB4 interaction failed sofar (Titapiwatanakun et al. 2009).

In summary, these data provide evidence for tissue-specific ABCB–PIN pairings that function interactively. In these, PIN proteins seem to add a vectorial dimension to ABCB-mediated non-polar cellular auxin export required for PAT. Functional interaction was supported by synergistic *abcb19 pin1* plant phenotypes and *abcb1 abcb19 pin2* root agravitropism (Blakeslee et al. 2007). Not surprisingly these

interactions correlate with their proposed overlapping functions in apical and basipetal auxin transport, respectively.

Beside heterodimerization of half-size transporters (McFarlane et al. 2010), no functional interaction between ABC transporters has been described in plants, but based on the above findings might be very likely.

Taken together, these findings suggest that the tendency of full-size ABC transporters to interact with one another and with other transporters appears more frequently than thought. Therefore, physical interactions between full-size transporters could serve as an important form of regulation and communication. Changes in the amount of one transporter, for example, in response to altered environmental conditions, would cause a shift in the binding equilibrium between transporters, acting as a means of rapidly altering transporter activity. It is tempting to speculate that ABC transporters may have a general role as functional regulators of membrane proteins in addition to their known transport roles. Further study of the plant ABC interactome will help elucidating whether such regulation is a common occurrence and what regulatory mechanisms are used.

7 Protein Phosphorylation

Protein phosphorylation is one of the most common and efficient mechanisms of post-translational transporter regulation in the cell. Putative phosphorylation sites have been identified in members of almost every ABC transporter subfamily from yeast to man (Stolarczyk et al. 2011). Therefore, it is reasonable to assume that all ABC transporters are regulated by phosphorylation because this mode provides cells with a simple, low energy, fast, and efficient way to change transporter function (Stolarczyk et al. 2011). In that respect, it is not surprising that abnormal phosphorylation is associated with many human diseases and conditions and many diseases occur as the result of mutated phosphorylation sites (Stolarczyk et al. 2011).

The linker region between NBD1 and MSD2 of ABCC7/CFTR is extensively phosphorylated and is thus referred to as the regulatory domain (R domain) and ABCC7 regulation via kinase-mediated phosphorylation is probably the most extensively characterized. ABCC7 function appears to be regulated by phosphorylation within NBD1 by PKC, and by extensive PKA- and PKC-mediated phosphorylation within the R domain (Dahan et al. 2001; Bozoky et al. 2013). Importantly, detailed analysis of the phosphorylation sites suggests that phosphorylation directly and indirectly is affected by previous phosphorylation events.

A combination of NMR, computational studies and three dimensional cryomicroscopy indicate that phosphorylation of the R domain results in a conformational change that pushes the R domain away from the core and NBDs which results in an increase in the apparent size/radius of the protein (Gadsby et al. 2006). Alternatively, it was suggested that R domain phosphorylation prevents “compacting” of the ABCC7 protein and opens the core to accept ions for transport

(Kanelis et al. 2010, 2011). These findings provide important insight into the overall role of phosphorylation in the regulation of all ABC transporters as similar domains are found in other ABC transporters and are referred to here as the "R-like" or "linker" domain.

Phosphorylation of ABCA1 implicated in cholesterol efflux and Tangier disease suggested a role for phosphorylation in the regulation of ABCA1 protein activity and stabilization/degradation. ABCC8/SUR1 and ABCC9/SUR2 are phosphorylated by inducing activation of the KATP channel (Giblin et al. 2002). However, the mechanism by which phosphorylation of ABCC8 and ABCC9 is determining the interaction and regulation of Kir6.2 and thus KATP channel activity is still unknown.

Several lines of clinical evidence suggest ABCBs as general targets for phosphorylation dependent regulation in a so-called linker region connecting the N- and C-terminal NBDs of ABCBs. It is now apparent that ABCB1 is likely phosphorylated by a number of kinases, including PKC and PKA, regulating the drug transport and ATPase properties (Szabo et al. 1997). However, a number of conflicting studies have been published as to the role of phosphorylation in regulating ABCB1 function and yet no clear consensus has been reached (Rumsby et al. 1998). It is important to note that a large number of studies have shown that many of the known inhibitors of PKA, PKC, and many other kinases are both substrates and/or inhibitors of ABCB1 function (Rumsby et al. 1998). There is increasing evidence that the same is true for some of the ABCC and ABCG subfamilies of transporters.

Employing different phospho-proteomic approaches, plant ABC proteins have recently shown to be phosphorylated (Nuhse et al. 2004; de la Fuente van Bentem et al. 2006; Peck 2006; Benschop et al. 2007) but the functional significance for only two ABCBs has been analyzed in detail.

The AGC4 kinase, PHOTROPIN1 (phot1) was shown to interact with both NBDs of ABCB19 but not with the NBD2 of ABCB1 (Christie et al. 2011). Using co-expression in HeLa cells, auxin efflux activity of ABCB19 but not of B1 was shown to be specifically inhibited by phot1 co-expression, in a mode that is dependent on the phot1 kinase activity and that is accelerated by light irradiation. However, although in vitro phosphorylation experiments verified ABCB19 as phot1 kinase substrate, phosphorylated domains and residues remained exclusive.

Using co-immunoprecipitation and LC-MS/MS analysis, the AGC3 kinase PINOID (PID) was identified as a valid partner in interaction with TWD1 (Henrichs et al. 2012). In vitro experiments and yeast expression analyses indicated that PID specifically modulates ABCB1-mediated auxin efflux in an action that is dependent on its kinase activity. Co-transfection in tobacco revealed that PID enhances ABCB1-mediated auxin efflux in the absence of TWD1, while PID had a negative impact on ABCB1 in yeast. Interestingly, triple ABCB1/PID/TWD1 co-transfection in tobacco revealed that PID blocks ABCB1-mediated auxin efflux in the presence of TWD1 (see Fig. 1), suggesting that TWD1 might function as a recruiting factor for ABCB1 phosphorylation. The fact that ABCB1 phosphorylation in the presence of TWD1 has the opposite effect on ABCB1 transport capacity

than TWD1–ABCB1 interaction per se argues for the idea that protein phosphorylation is not the primary mode of TWD1 activation. Obviously, both modes of ABCB1 regulation—directly via TWD1 interaction or by PID phosphorylation—might also take place in parallel or in competition, resulting in fine-tuning of ABCB activity as reported for mammalian ABCBs. Alternatively, ABCB1 phosphorylation in the presence of TWD1 might disrupt TWD1–ABCB1 interaction leading to ABCB1 inhibition (see below).

S634 was identified as a key residue of the regulatory ABCB1 linker, which was verified by mutation analyses in yeast and tobacco (Henrichs et al. 2012). In the absence of TWD1, PID does phosphorylate S634, resulting in ABCB1 activation. On the other hand, negative ABCB1 regulation in the presence of TWD1 argues for a second, PID-specific ABCB1 phosphorylation site that does not essentially need to be part of the linker.

In summary, these two analogous sets of data on ABCB19 and ABCB1 regulation by AGC kinases, phot1 (Christie et al. 2011) and PID (Henrichs et al. 2012), imply that AGC kinases have a direct impact on auxin efflux (ABCB) activity. Although phosphorylated residues in ABCB19 by phot1 have not yet been identified and a phot1-TWD1 interaction has not been proven (see Fig. 2), indicate an analogous mode of action: in the presence of TWD1, phosphorylation events catalyzed by phot1 and PID lead to an inhibition of ABCB activity. However, currently it is unclear if ABCB phosphorylation alters ABCB–TWD1 interaction, which would be a plausible ratio for a loss of functionality.

In conclusion, although the development of highly sensitive phosphoproteomic techniques has greatly improved our ability to identify sites of ABC transporter phosphorylation, it remained extremely difficult to investigate the impact of these phosphorylation sites by biochemical means. Therefore alternative tools, especially structure modeling tools are available to further address how phosphorylation regulates ABC transporter function. Finally, the identification of kinases (and phosphatases) that regulate ABC transporter function represents another major challenge but work on plant ABCB might suggest that protein–protein interaction analyses may aid in the development of novel kinase inhibitor-based therapies that will more effectively treat ABC transporter-related diseases.

8 Modulatory Drugs

MDR reversal agents (also termed chemosensitizers) are modulatory drugs that bock ABC transporter activity by (1) direct interaction with the ATP-binding site, drug binding sites or other ABCB domains that are critical for its transport function; (2) modulation of *ABCB* expression; (3) modulation of ABCB activity via inhibition of its post-translational modifications; and (4) indirect modulation of ABCB transport activities by other systems, such as regulatory components (Shapiro and Ling 1995). They comprise a diverse group of substances, which includes calcium and sodium channel blockers, calmodulin antagonists, local anesthetics, steroidal

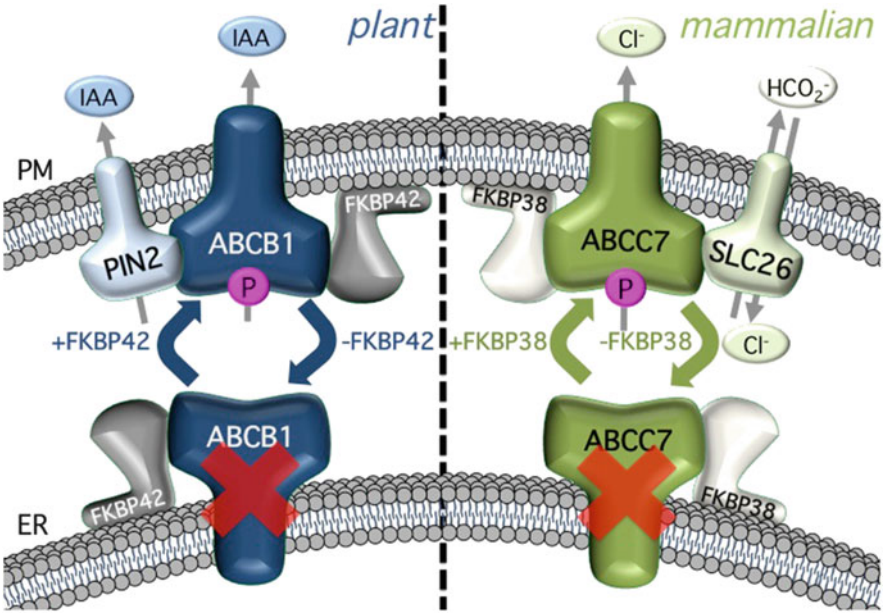

Fig. 2 Comparison of regulation of human ABCC7/CFTR and *Arabidopsis* ABCB1/PGP1 by FKBPs. The presence and auxin (IAA) export activity of ABCB1 is controlled by functional interaction between FKBP42/TWD1 and ABCB1 diagnosed on the plasma membrane (PM). ABCB1 functionally interacts with PIN-like, secondary active auxin exporters (exemplified by PIN2). In the absence of FKBP42/TWD1 (-FKBP42), ABCB1 is retained on the ER and consecutively degraded suggesting a chaperon-like function for FKBP42/TWD1. In analogy, the human ortholog of FKBP42, FKBP38, was shown to chaperone ABCC7/CFTR to the PM, where it functionally interacts with bicarbonate (HCO_3^-)-chloride (Cl^-) exchanger, SLC26, leading to enhanced bicarbonate and thus fluid secretion. Note that CFTR–SLC26 interaction is promoted by PDZ protein binding the C-termini of both partners (not shown here), which has not been shown for ABCBs. Further, that phosphorylation of both linker and R-domains of ABCB1 and ABCC7 by the AGC kinase, PINOID, and PKA, respectively, was shown to affect transport activity, while so far only for ABCC7–SLC26 interaction was shown to be enhanced

and structurally related compounds, immunosuppressive agents, protein kinase inhibitors, detergents, indole alkaloids, and macrolide compounds (Breier et al. 2013). Analyses of the individual mode of actions of these modulatory drugs are often hindered by the fact that many of these modulatory drugs have often an effect on several of the mechanisms listed above and are even often themselves ABCB substrates.

Considerable efforts have been undertaken to identify new chemosensitizing agents for better treatment of human cancers. In that respect, flavonoids represent an interesting group of plant polyphenols that are found frequently in our daily diets and herbal products. Flavonols, such as quercetin and kaempferol, mainly present in leafy vegetables, are the most abundant flavonoids in foods. Naturally occurring flavonoids are glycosylated but aglycones are more potent antioxidants than their

corresponding glycosides (Williams et al. 2004). High intake of flavonoid compounds has been associated with a variety of human health benefits, including prevention of cancer, cardiovascular diseases, and osteoporosis.

Various mechanisms for the interaction of ABC transporters with flavonoids have been reported: a number of studies have demonstrated inhibition of ABC transporters by flavonoids (Morris and Zhang 2006) probably by mimicking ATP and competing for ABCB nucleotide binding domains (Conseil et al. 1998). On the other hand, flavonoids are themselves substrates of ABC transporters, making them competitive inhibitors toward other substrates (Morris and Zhang 2006). Moreover, some studies have even reported a stimulation of ABC transport activity. Therefore, finding a way to avoid harmful drug–flavonoid interactions mediated by these transporters has proven difficult. Nevertheless, flavonoids, such as genistein and quercetin, were shown to block ABCB1/PGP1 and BCRP transport, thereby increasing the intracellular accumulation of anticancer drugs, such as rhodamine 123, daunorubicin, paclitaxel, and vinblastine, making them promising candidates for co-administration with anticancer drugs.

Recently non-biochemical, in silico approaches have been shown to isolate modulatory drugs that have the potential to interfere with ABC transporter activities or even to revert mutational loss-of-function. In a very interesting structure-based virtual screening to identify new low-molecular-weight compounds that bind to $CFTR^{\Delta F508}$, a compound was identified and shown to correct for the $CFTR^{\Delta F508}$ trafficking defect. Interestingly, the compound was shown to bind to NBD1 of $CFTR^{\Delta F508}$ but also to disrupt keratin8-$CFTR^{\Delta F508}$ interaction (Odolczyk et al. 2013).

Flavonoids were suggested to act as PAT regulators, initially based on their ability to compete with NPA for transporter binding sites (Brown et al. 2001). This concept was further supported by auxin-related phenotypes of *Arabidopsis* mutants with altered flavonoid levels (Buer and Djordjevic 2009), although fundamental physiological and developmental processes occur in the absence of flavonoids. Aglycone molecules, such as quercetin and kaempferol, have been shown to inhibit PAT and consequently to enhance localized auxin accumulation (Brown et al. 2001). Currently they are seen as transport regulators or modulators (Peer and Murphy 2007); nevertheless, the mechanisms by which flavonoids interfere with auxin efflux components were for a long time unclear.

Things are complicated by the finding that aglycone flavonoids, like naringenin, dihydro-kaempferol, and dihydro-quercetin are taken up at the root tip and travel long distances via cell-to-cell movement to distal tissues, followed by conversion to quercetin and kaempferol (Buer et al. 2007). Usage of inhibitors suggested that ABCC transporter facilitated flavonoid movement away from the application site. Indeed, ABCC were suggested as vacuolar importers for glycosylated flavonoids, however, their molecular identity remains unknown (Klein et al. 2006).

In analogy to mammalian ABC transporters, ABCB1, ABCB4 and ABCB19 were shown to be inhibited by nM concentrations of the aglycone flavonol, querectin, while uM NPA concentrations were needed to cause a similar effect (Geisler et al. 2005; Terasaka et al. 2005; Bailly et al. 2008). Together with the

finding that ABCB1 and ABCB19 (jointly with TWD1) have been identified by NPA chromatography (Noh et al. 2001; Murphy et al. 2002; Geisler et al. 2003) and shown to bind NPA (Kim et al. 2010; Bailly et al. 2008; Rojas-Pierce et al. 2007) this indicates that ABCB-type auxin transporters are inhibited by NPA binding.

By means of chemical genomics, another auxin efflux inhibitor, termed BUM (2-[4-(diethylamino)-2-hydroxybenzoyl]benzoic acid), was identified by its potential to efficiently block auxin-regulated plant physiology and development (Kim et al. 2010). Based on computational binding, NPA was suggested to dock to pockets that are flanked by coupling helices and Q loops of NBD1 and NBD2 at the NBD-ICL interface (Kim et al. 2010). Mutational analyses of these regions supported the idea that NPA blocks efficiently the main mechanistic of the transporter during transition of conformational changes between the NBDs and the ICLs.

Interestingly, using a yeast-based BRET (Bioluminescence resonance energy transfer) system NPA and BUM, but not competitive ATIs (such as TIBA or CPD), were shown to disrupt TWD1–ABCB1 interaction (Bailly et al. 2008; Kim et al. 2010). Further, all flavonoids tested disrupted the interaction as well with the flavonol, quercetin, being the most efficient. Mutant analysis indicated that the TWD1 FKBD is responsible for both interaction and drug regulation of ABCB1. Interestingly, the TWD1 FKBD binds NPA but not quercetin itself, suggesting that disruption is caused by quercetin binding to ABCBs (Bailly et al. 2008; Kim et al. 2010).

These data suggest a novel mode of drug-mediated regulation of ABCB activity via an interacting FKBP. The TWD1 FKBD owns a receptor-like function and is therefore capable of integrating negative (ATI) inputs on ABCB1 (see Fig. 3). In silico docking of NPA on the crystal structure of the FKBD provides indication for an ATI-binding pocket that is interestingly overlapping with surfaces thought to dock to the ABCB1 NBD2 (Granzin et al. 2006) providing a mechanistic ratio for disruption of TWD1–ABCB interaction.

Although individual binding affinities for TWD1 and ABCBs have not yet been determined, these data are in agreement with the current concept that the efflux complex consists of at least two proteins: a membrane-embedded low-affinity (transporter) and a peripheral, high-affinity NPA-binding regulatory subunit (Morris 2000; Luschnig 2001; Petrasek et al. 2003).

9 ABC Transporter Interaction, Phosphorylation, and Secretion are Interconnected by the FKBP42, TWISTED DWARF1

A whole series of data imply that plant ABCB activity is regulated by interconnected feedback-loops (see Fig. 3). In a protein–protein interaction loop, distinct ABCB–PIN and ABCB–TWD1 pairings of a putative multiprotein auxin efflux complex are building the basis for a plastic control of auxin streams during

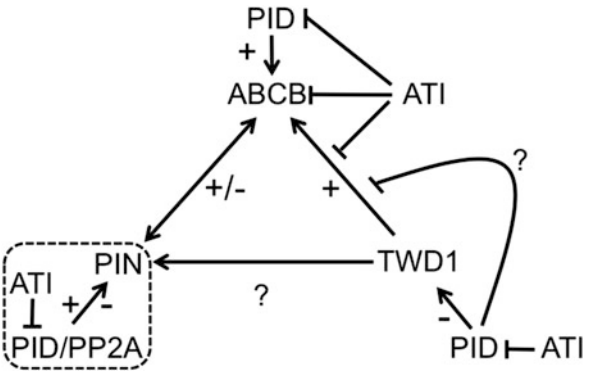

Fig. 3 FKBP42/TWD1 is the central element of ABCB regulation. In a protein–protein interaction loop, TWD1 and PIN proteins positively (+) contribute to ABCB-mediated auxin transport by protein–protein interaction. ATIs, such as flavonols, disrupt ABCB1–TWD1 interaction probably by binding to ABCB1 resulting in transport inhibition. *Arrows* denote positive and *bars* negative regulation at the transport level. In a polarity loop (*dashed line*; Benjamins and Scheres 2008), PIN polarity is coordinated by the phosphorylation status of PINs controlled by PID/PP2A action. Moreover, PID was shown to control also PIN and ABCB transport activity; the latter involves interaction with TWD1. Note that a negative impact of PID on TWD1–ABCB1 interaction by ABCB1 phosphorylation has not yet been demonstrated. Figure modified from Geisler and Henrichs (2013)

PAT (Geisler et al. 2014). Currently it is unclear if this includes a direct TWD1–PIN interaction, which was suggested based on the finding that in planta BRET analyses revealed a weak but significant PIN1–TWD1 interaction (Wang et al. 2013). However, in contrast to ABCBs expression and locatlization, PIN1 and PIN2 are unchanged in *twd1* (Bouchard et al. 2006; Wu et al. 2010).

Recent findings now suggest that ABCB interaction and PIN polarity loops (Benjamins and Scheres 2008) are interconnected via the action of AGC kinases, exemplified here by PID (Fig. 3): beside decoding PIN polarity (Michniewicz et al. 2007) and regulating PIN activity (Wang et al. 2012), PID also interacts with TWD1, thus negatively regulating ABCB1 activity (Henrichs et al. 2012).

Interestingly, modulatory drugs that act as ATIs, such as quercetin (but not NPA), are able to interfere with both interaction and polarity loops on different levels: by (1) direct inhibition of ABCB activity, (2) disrupting TWD1–ABCB interaction, and (3) inhibition of PID itself has an indirect impact on ABCB1 phosphorylation/activity or PIN polarity. As such, these partially opposite effects reflect pretty well the complexity of flavonol action on ABCB activity, which might result from combinatory effects on ABCB activity, its interaction with TWD1 and PID and additionally on PIN gene expression and cellular trafficking (Peer et al. 2001, 2004; Peer and Murphy 2006; Santelia et al. 2008).

In summary it appears that individual ABCBs and regulatory proteins interact interfere and regulate each other in order to allow a fine-tuning of ABCB-mediated auxin distribution pattern. TWD1 is apparently the central and integrative element of ABCB PPI and phosphorylation feedback loops controlling ABCB activity:

TWD1 directly interacts and stabilizes as a chaperone ABCB ER-to-PM secretion (Wu et al. 2010; Wang et al. 2013) and controls by recruiting the AGC kinase, PID, the phosphorylation status and thus activity of ABCB1. As such, especially in light of the unequal ABCB–TWD1 stoichiometry, initial models of fixed ABCB–TWD1 complexes (Geisler et al. 2003) have become less unlikely. However a transient ABCB–TWD1 interaction on the PM and a chaperone-like role for TWD1 is perfectly inline with the suggestion that the membrane-attached NPA-binding protein is required for auxin efflux transporter positioning (Gil et al. 2001).

However, at present it is unclear if ABCB1 phosphorylation by PID in the presence of TWD1 is causing disruption of TWD1–ABCB interaction. Further, it is currently unknown if ABCB phosphorylation by PID has an influence on ABCB secretion or protein stability.

10 Conclusion and Outlook

In this chapter we have compared the recent knowledge on plant and non-plant post-transcriptional ABC transporter regulation. Despite the limited information on plant ABC regulation it becomes more and more evident that many regulatory mechanisms—especially on the level of PPI, protein phosphorylation and protein secretion—found for mammalian ABC transporters are also conserved in plants. We have focused our attention in more detail on members of the Arabidopsis ABCB that function in vivo as specific transporters of the plant hormone auxin, and that in respect to their regulation have been analyzed in greater detail.

As pointed out above, the FKBP, TWD1, is a central element of ABCB regulation. A comparison with the functionality of the human ortholog of TWD1, FKBP38, indicates that many features, like degradation of their targets in their absence, are conserved between ABCC7/CFTR (see Fig. 2) and ABCB1/PGP1, although both transporters belong to different subclasses. In this respect it is worth to recall that TWD1 interacts and thus might regulate also ABCC-like transporters on the plant vacuole (Geisler et al. 2004).

This dual activity on two different organelles is also found for human FKBP38 that besides acting as CFTR chaperon, functions as a scavenger of anti-apoptotic proteins on the mitochondrial outer membrane (Shirane and Nakayama 2003). Interestingly, both anti-apoptotic function of HsFKBP38 and CFTR regulation are under control by calmodulin activating the *cis–trans* peptidyl-prolyl isomerase (PPIase) activity of HsFKBP38 (Edlich et al. 2005, 2007). Taking the human FKBP38 as a paradigm, it seems likely that a steric change of ABCBs (or interacting AGC kinases) is induced by calcium-dependent binding of calmodulin-like proteins that might affect directly or indirectly (via PID) ABCB activity. However, such a PPIase activity has been proven to be absent in TWD1 (Geisler et al. 2003), suggesting a different mode of action.

On the other hand, the role of FKBP38-mediated recruitment of CFTR phosphorylating kinases, such as PKA or PKC, has not yet been investigated. Likewise,

the physical interaction between CFTR and FKBP38 awaits to our knowledge still its confirmation.

Moreover, decades of auxin transport research have provided us with a set of highly potent ABCB inhibitors that block at least plant ABCBs at the sub-micromolar level. Unfortunately, these have not yet been tested for their potential as inhibitors of mammalian ABCBs.

Finally, using an evolutionary-structural approach recently evidence was provided why plant ABCBs own a higher degree of substrate specificity in comparison to their mammalian counterparts: outward-facing amino acids of putative auxin hotspots in the central cavity formed by transmembrane helices overlap with known mammalian substrate-binding domains but show a plant-kingdom-specific, high degree of conservation (Bailly et al. 2011). Related to this it was shown that TWD1 does not only modulate ABCB1 activity but also substrate specificity. Loss-of *TWD1* in Arabidopsis affects IAA but not NAA export, while co-expression of ABCB1 with TWD1 in yeast affects IAA but not NAA export (Bailly et al. 2013). The underlying mechanism is unclear but two plausible causalities might be of interest for clinical ABCB research: first, loss-of substrate specificity in cancer lines might be caused by massive over-production of ABCB resulting in an obvious mismatch in the ABCB–FKBP ratio, which again might lead to unspecific ABCBs and thus MDR. And second, and related to the first point, interaction of TWD1/FKBP42 with the NBD2 of ABCB1 might cause an unequal inhibition of NBDs inside the ABCB molecule that might correlate with a change in substrate specificity.

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References

Ambudkar SV, Kimchi-Sarfaty C, Sauna ZE, Gottesman MM (2003) P-glycoprotein: from genomics to mechanism. Oncogene 22:7468–7485

Bacso Z, Nagy H, Goda K, Bene L, Fenyvesi F, Matko J, Szabo G (2004) Raft and cytoskeleton associations of an ABC transporter: P-glycoprotein. Cytometry A 61:105–116

Bailly A, Yang H, Martinoia E, Geisler M, Murphy AS (2011) Plant lessons: exploring ABCB functionality through structural modeling. Front Plant Sci 2:108

Bailly A, Sovero V, Vincenzetti V, Santelia D, Bartnik D, Koenig BW, Mancuso S, Martinoia E, Geisler M (2008) Modulation of P-glycoproteins by auxin transport inhibitors is mediated by interaction with immunophilins. J Biol Chem 283:21817–21826

Bailly A, Wang B, Zwiewka M, Pollmann S, Schenck D, Luthen H, Schulz A, Friml J, Geisler M (2013) Expression of TWISTED DWARF1 lacking its in-plane membrane anchor leads to increased cell elongation and hypermorphic growth. Plant J 77:108–118

Banasavadi-Siddegowda YK, Mai J, Fan Y, Bhattacharya S, Giovannucci DR, Sanchez ER, Fischer G, Wang X (2011) FKBP38 peptidylprolyl isomerase promotes the folding of cystic fibrosis transmembrane conductance regulator in the endoplasmic reticulum. J Biol Chem 286:43071–43080

Benjamins R, Scheres B (2008) Auxin: the looping star in plant development. Annu Rev Plant Biol 59:443–465

Benschop JJ, Mohammed S, O'Flaherty M, Heck AJ, Slijper M, Menke FL (2007) Quantitative phosphoproteomics of early elicitor signaling in Arabidopsis. Mol Cell Proteomics 6: 1198–1214

Bessadok A, Garcia E, Jacquet H, Martin S, Garrigues A, Loiseau N, Andre F, Orlowski S, Vivaudou M (2011) Recognition of sulfonylurea receptor (ABCC8/9) ligands by the multidrug resistance transporter P-glycoprotein (ABCB1): functional similarities based on common structural features between two multispecific ABC proteins. J Biol Chem 286:3552–3569

Blakeslee JJ, Bandyopadhyay A, Lee OR, Mravec J, Titapiwatanakun B, Sauer M, Makam SN, Cheng Y, Bouchard R, Adamec J, Geisler M, Nagashima A, Sakai T, Martinoia E, Friml J, Peer WA, Murphy AS (2007) Interactions among PIN-FORMED and P-glycoprotein auxin transporters in Arabidopsis. Plant Cell 19:131–147

Borner GH, Sherrier DJ, Weimar T, Michaelson LV, Hawkins ND, Macaskill A, Napier JA, Beale MH, Lilley KS, Dupree P (2005) Analysis of detergent-resistant membranes in Arabidopsis. Evidence for plasma membrane lipid rafts. Plant Physiol 137:104–116

Bouchard R, Bailly A, Blakeslee JJ, Oehring SC, Vincenzetti V, Lee OR, Paponov I, Palme K, Mancuso S, Murphy AS, Schulz B, Geisler M (2006) Immunophilin-like TWISTED DWARF1 modulates auxin efflux activities of Arabidopsis P-glycoproteins. J Biol Chem 281: 30603–30612

Bozoky Z, Krzeminski M, Chong PA, Forman-Kay JD (2013) Structural changes of CFTR R region upon phosphorylation: a plastic platform for intramolecular and intermolecular interactions. FEBS J 280:4407–4416

Breier A, Gibalova L, Seres M, Barancik M, Sulova Z (2013) New insight into p-glycoprotein as a drug target. Anticancer Agents Med Chem 13:159–170

Brown DE, Rashotte AM, Murphy AS, Normanly J, Tague BW, Peer WA, Taiz L, Muday GK (2001) Flavonoids act as negative regulators of auxin transport in vivo in arabidopsis. Plant Physiol 126:524–535

Buer CS, Djordjevic MA (2009) Architectural phenotypes in the transparent testa mutants of Arabidopsis thaliana. J Exp Bot 60:751–763

Buer CS, Muday GK, Djordjevic MA (2007) Flavonoids are differentially taken up and transported long distances in Arabidopsis. Plant Physiol 145:478–490

Burgardt NI, Linnert M, Weiwad M, Geisler M, Lucke C (2012) NMR assignments of the FKBP-type PPIase domain of FKBP42 from Arabidopsis thaliana. Biomol NMR Assign 6:185–188

Calcagno AM, Kim IW, Wu CP, Shukla S, Ambudkar SV (2007) ABC drug transporters as molecular targets for the prevention of multidrug resistance and drug-drug interactions. Curr Drug Deliv 4:324–333

Cameron AM, Steiner JP, Sabatini DM, Kaplin AI, Walensky LD, Snyder SH (1995) Immunophilin FK506 binding protein associated with inositol 1,4,5-trisphosphate receptor modulates calcium flux. Proc Natl Acad Sci U S A 92:1784–1788

Cant N, Pollock N, Ford RC (2014) CFTR structure and cystic fibrosis. Int J Biochem Cell Biol. doi:10.1016/j.biocel.2014.02.004

Cho M, Cho HT (2012) The function of ABCB transporters in auxin transport. Plant Signal Behav 8

Christie JM, Yang H, Richter GL, Sullivan S, Thomson CE, Lin J, Titapiwatanakun B, Ennis M, Kaiserli E, Lee OR, Adamec J, Peer WA, Murphy AS (2011) phot1 inhibition of ABCB19 primes lateral auxin fluxes in the shoot apex required for phototropism. PLoS Biol 9:e1001076

Conseil G, Baubichon-Cortay H, Dayan G, Jault JM, Barron D, Di Pietro A (1998) Flavonoids: a class of modulators with bifunctional interactions at vicinal ATP- and steroid-binding sites on mouse P-glycoprotein. Proc Natl Acad Sci U S A 95:9831–9836

Czar MJ, Galigniana MD, Silverstein AM, Pratt WB (1997) Geldanamycin, a heat shock protein 90-binding benzoquinone ansamycin, inhibits steroid-dependent translocation of the glucocorticoid receptor from the cytoplasm to the nucleus. Biochemistry 36:7776–7785

Dahan D, Evagelidis A, Hanrahan JW, Hinkson DA, Jia Y, Luo J, Zhu T (2001) Regulation of the CFTR channel by phosphorylation. Pflugers Archiv 443(Suppl 1):S92–96

Dawson RJ, Locher KP (2007) Structure of the multidrug ABC transporter Sav 1866 from Staphylococcus aureus in complex with AMP-PNP. FEBS Lett 581:935–938

de la Fuente van Bentem S, Anrather D, Roitinger E, Djamei A, Hufnagl T, Barta A, Csaszar E, Dohnal I, Lecourieux D, Hirt H (2006) Phosphoproteomics reveals extensive in vivo phosphorylation of Arabidopsis proteins involved in RNA metabolism. Nucleic Acids Res 34: 3267–3278

Diop NK, Hrycyna CA (2005) N-Linked glycosylation of the human ABC transporter ABCG2 on asparagine 596 is not essential for expression, transport activity, or trafficking to the plasma membrane. Biochemistry 44:5420–5429

Dos Santos SM, Weber CC, Franke C, Muller WE, Eckert GP (2007) Cholesterol: coupling between membrane microenvironment and ABC transporter activity. Biochem Biophys Res Commun 354:216–221

Edlich F, Lucke C (2011) From cell death to viral replication: the diverse functions of the membrane-associated FKBP38. Curr Opin Pharmacol 11:1–6

Edlich F, Weiwad M, Erdmann F, Fanghanel J, Jarczowski F, Rahfeld JU, Fischer G (2005) Bcl-2 regulator FKBP38 is activated by Ca2+/calmodulin. EMBO J 24:2688–2699

Edlich F, Maestre-Martinez M, Jarczowski F, Weiwad M, Moutty MC, Malesevic M, Jahreis G, Fischer G, Lucke C (2007) A novel calmodulin-Ca2+ target recognition activates the Bcl-2 regulator FKBP38. J Biol Chem 282:36496–36504

Feraru E, Friml J (2008) PIN polar targeting. Plant Physiol 147:1553–1559

Gadsby DC, Vergani P, Csanady L (2006) The ABC protein turned chloride channel whose failure causes cystic fibrosis. Nature 440:477–483

Geisler M, Murphy AS (2006) The ABC of auxin transport: the role of p-glycoproteins in plant development. FEBS Lett 580:1094–1102

Geisler M, Bailly A (2007) Tete-a-tete: the function of FKBPs in plant development. Trends Plant Sci 12:465–473

Geisler M, Bailly A (2008) Tête-à-tête: FKBPs function as key players in plant development. Trends Plant Sci 12:465–73

Geisler M, Henrichs S (2013) Regulation of polar auxin transport by protein-protein interactions. In: Chen R, Baluška F (eds) Signaling and communication in polar auxin transport. Springer, Berlin

Geisler M, Wang B, Zhu J (2014) Auxin transport during root gravitropism: transporters and techniques. Plant Biol 16(Suppl 1):50–57

Geisler M, Girin M, Brandt S, Vincenzetti V, Plaza S, Paris N, Kobae Y, Maeshima M, Billion K, Kolukisaoglu UH, Schulz B, Martinoia E (2004) Arabidopsis immunophilin-like TWD1 functionally interacts with vacuolar ABC transporters. Mol Biol Cell 15:3393–3405

Geisler M, Kolukisaoglu HU, Bouchard R, Billion K, Berger J, Saal B, Frangne N, Koncz-Kalman Z, Koncz C, Dudler R, Blakeslee JJ, Murphy AS, Martinoia E, Schulz B (2003) TWISTED DWARF1, a unique plasma membrane-anchored immunophilin-like protein, interacts with Arabidopsis multidrug resistance-like transporters AtPGP1 and AtPGP19. Mol Biol Cell 14:4238–4249

Geisler M, Blakeslee JJ, Bouchard R, Lee OR, Vincenzetti V, Bandyopadhyay A, Titapiwatanakun B, Peer WA, Bailly A, Richards EL, Ejendal KF, Smith AP, Baroux C, Grossniklaus U, Muller A, Hrycyna CA, Dudler R, Murphy AS, Martinoia E (2005) Cellular efflux of auxin catalyzed by the Arabidopsis MDR/PGP transporter AtPGP1. Plant J 44:179–194

Geldner N, Richter S, Vieten A, Marquardt S, Torres-Ruiz RA, Mayer U, Jurgens G (2004) Partial loss-of-function alleles reveal a role for GNOM in auxin transport-related, post-embryonic development of Arabidopsis. Development 131:389–400

Giblin JP, Quinn K, Tinker A (2002) The cytoplasmic C-terminus of the sulfonylurea receptor is important for KATP channel function but is not key for complex assembly or trafficking. Eur J Biochem 269:5303–5313

Gil P, Dewey E, Friml J, Zhao Y, Snowden KC, Putterill J, Palme K, Estelle M, Chory J (2001) BIG: a calossin-like protein required for polar auxin transport in Arabidopsis. Genes Dev 15: 1985–1997

Granzin J, Eckhoff A, Weiergraber OH (2006) Crystal structure of a multi-domain immunophilin from Arabidopsis thaliana: a paradigm for regulation of plant ABC transporters. J Mol Biol 364:799–809

Hemenway CS, Heitman J (1996) Immunosuppressant target protein FKBP12 is required for P-glycoprotein function in yeast. J Biol Chem 271:18527–18534

Henrichs S, Wang B, Fukao Y, Zhu J, Charrier L, Bailly A, Oehring SC, Linnert M, Weiwad M, Endler A, Nanni P, Pollmann S, Mancuso S, Schulz A, Geisler M (2012) Regulation of ABCB1/PGP1-catalysed auxin transport by linker phosphorylation. EMBO J 31:2965–80. doi:10.1038/emboj.2012.120

Higgins CF (2007) Multiple molecular mechanisms for multidrug resistance transporters. Nature 446:749–757

Ismair MG, Hausler S, Stuermer CA, Guyot C, Meier PJ, Roth J, Stieger B (2009) ABC-transporters are localized in caveolin-1-positive and reggie-1-negative and reggie-2-negative microdomains of the canalicular membrane in rat hepatocytes. Hepatology 49: 1673–1682

Jasinski M, Ducos E, Martinoia E, Boutry M (2003) The ATP-binding cassette transporters: structure, function, and gene family comparison between rice and Arabidopsis. Plant Physiol 131:1169–1177

Jurgens G, Geldner N (2007) The high road and the low road: trafficking choices in plants. Cell 130:977–979

Kamimoto Y, Terasaka K, Hamamoto M, Takanashi K, Fukuda S, Shitan N, Sugiyama A, Suzuki H, Shibata D, Wang B, Pollmann S, Geisler M, Yazaki K (2012) Arabidopsis ABCB21 is a facultative auxin importer/exporter regulated by cytoplasmic auxin concentration. Plant Cell Physiol 53:2090–2100

Kamphausen T, Fanghanel J, Neumann D, Schulz B, Rahfeld JU (2002) Characterization of Arabidopsis thaliana AtFKBP42 that is membrane-bound and interacts with Hsp90. Plant J 32:263–276

Kanelis V, Chong PA, Forman-Kay JD (2011) NMR spectroscopy to study the dynamics and interactions of CFTR. Methods Mol Biol 741:377–403

Kanelis V, Hudson RP, Thibodeau PH, Thomas PJ, Forman-Kay JD (2010) NMR evidence for differential phosphorylation-dependent interactions in WT and DeltaF508 CFTR. EMBO J 29:263–277

Kang J, Hwang JU, Lee M, Kim YY, Assmann SM, Martinoia E, Lee Y (2010) PDR-type ABC transporter mediates cellular uptake of the phytohormone abscisic acid. Proc Natl Acad Sci U S A 107:2355–2360

Kepinski S, Leyser O (2005) Plant development: auxin in loops. Curr Biol 15:R208–210

Kim JY, Henrichs S, Bailly A, Vincenzetti V, Sovero V, Mancuso S, Pollmann S, Kim D, Geisler M, Nam HG (2010) Identification of an ABCB/P-glycoprotein-specific inhibitor of auxin transport by chemical genomics. J Biol Chem 285:23309–23317

Kipp H, Arias IM (2000) Intracellular trafficking and regulation of canalicular ATP-binding cassette transporters. Semin Liver Dis 20:339–351

Klappe K, Hummel I, Hoekstra D, Kok JW (2009) Lipid dependence of ABC transporter localization and function. Chem Phys Lipids 161:57–64

Klein M, Burla B, Martinoia E (2006) The multidrug resistance-associated protein (MRP/ABCC) subfamily of ATP-binding cassette transporters in plants. FEBS Lett 580:1112–1122

Klein M, Perfus-Barbeoch L, Frelet A, Gaedeke N, Reinhardt D, Mueller-Roeber B, Martinoia E, Forestier C (2003) The plant multidrug resistance ABC transporter AtMRP5 is involved in guard cell hormonal signalling and water use. Plant J 33:119–129

Kleine-Vehn J, Friml J (2008) Polar targeting and endocytic recycling in auxin-dependent plant development. Annu Rev Cell Dev Biol 24:447–473

Kretzschmar T, Kohlen W, Sasse J, Borghi L, Schlegel M, Bachelier JB, Reinhardt D, Bours R, Bouwmeester HJ, Martinoia E (2012) A petunia ABC protein controls strigolactone-dependent symbiotic signalling and branching. Nature 483:341–344

Kuromori T, Miyaji T, Yabuuchi H, Shimizu H, Sugimoto E, Kamiya A, Moriyama Y, Shinozaki K (2010) ABC transporter AtABCG25 is involved in abscisic acid transport and responses. Proc Natl Acad Sci U S A 107:2361–2366

Lalonde S, Ehrhardt DW, Loque D, Chen J, Rhee SY, Frommer WB (2008) Molecular and cellular approaches for the detection of protein-protein interactions: latest techniques and current limitations. Plant J 53:610–635

Lalonde S, Sero A, Pratelli R, Pilot G, Chen J, Sardi MI, Parsa SA, Kim DY, Acharya BR, Stein EV, Hu HC, Villiers F, Takeda K, Yang Y, Han YS, Schwacke R, Chiang W, Kato N, Loque D, Assmann SM, Kwak JM, Schroeder JI, Rhee SY, Frommer WB (2010) A membrane protein/signaling protein interaction network for Arabidopsis version AMPv2. Front Physiol 1:24

Lam P, Xu S, Soroka CJ, Boyer JL (2012) A C-terminal tyrosine-based motif in the bile salt export pump directs clathrin-dependent endocytosis. Hepatology 55:1901–1911

Langowski L, Ruzicka K, Naramoto S, Kleine-Vehn J, Friml J (2010) Trafficking to the outer polar domain defines the root-soil interface. Curr Biol 20:904–908

Laude AJ, Prior IA (2004) Plasma membrane microdomains: organization, function and trafficking. Mol Membr Biol 21:193–205

Leonhardt N, Vavasseur A, Forestier C (1999) ATP binding cassette modulators control abscisic acid-regulated slow anion channels in guard cells. Plant Cell 11:1141–1152

Li C, Naren AP (2011) Analysis of CFTR interactome in the macromolecular complexes. Methods Mol Biol 741:255–270

Li Y, Prinz WA (2004) ATP-binding cassette (ABC) transporters mediate nonvesicular, raft-modulated sterol movement from the plasma membrane to the endoplasmic reticulum. J Biol Chem 279:45226–45234

Lievens S, Eyckerman S, Lemmens I, Tavernier J (2010) Large-scale protein interactome mapping: strategies and opportunities. Expert Rev Proteomics 7:679–690

Linton KJ (2007) Structure and function of ABC transporters. Physiology (Bethesda) 22:122–130

Loo TW, Clarke DM (1997) Identification of residues in the drug-binding site of human P-glycoprotein using a thiol-reactive substrate. J Biol Chem 272:31945–31948

Luschnig C (2001) Auxin transport: why plants like to think BIG. Curr Biol 11:R831–833

Martinoia E, Klein M, Geisler M, Bovet L, Forestier C, Kolukisaoglu U, Muller-Rober B, Schulz B (2002) Multifunctionality of plant ABC transporters–more than just detoxifiers. Planta 214: 345–355

McFarlane HE, Shin JJ, Bird DA, Samuels AL (2010) Arabidopsis ABCG transporters, which are required for export of diverse cuticular lipids, dimerize in different combinations. Plant Cell 22:3066–3075

Michniewicz M, Zago MK, Abas L, Weijers D, Schweighofer A, Meskiene I, Heisler MG, Ohno C, Zhang J, Huang F, Schwab R, Weigel D, Meyerowitz EM, Luschnig C, Offringa R, Friml J (2007) Antagonistic regulation of PIN phosphorylation by PP2A and PINOID directs auxin flux. Cell 130:1044–1056

Miller G (2006) Scientific publishing. A scientist's nightmare: software problem leads to five retractions. Science 314:1856–1857

Modok S, Mellor HR, Callaghan R (2006) Modulation of multidrug resistance efflux pump activity to overcome chemoresistance in cancer. Curr Opin Pharmacol 6:350–354

Mohrmann K, van Eijndhoven MA, Schinkel AH, Schellens JH (2005) Absence of N-linked glycosylation does not affect plasma membrane localization of breast cancer resistance protein (BCRP/ABCG2). Cancer Chemother Pharmacol 56:344–350

Monterisi S, Casavola V, Zaccolo M (2013) Local modulation of cystic fibrosis conductance regulator: cytoskeleton and compartmentalized cAMP signalling. Br J Pharmacol 169:1–9

Morita M, Imanaka T (2012) Peroxisomal ABC transporters: structure, function and role in disease. Biochim Biophys Acta 1822:1387–1396

Morris DA (2000) Transmembrane auxin carrier systems–dynamic regulators of polar auxin transport. Plant Growth Regul 32:161–172

Morris ME, Zhang S (2006) Flavonoid-drug interactions: effects of flavonoids on ABC transporters. Life Sci 78:2116–2130

Murphy AS, Hoogner KR, Peer WA, Taiz L (2002) Identification, purification, and molecular cloning of N-1-naphthylphthalmic acid-binding plasma membrane-associated aminopeptidases from Arabidopsis. Plant Physiol 128:935–950

Noh B, Murphy AS, Spalding EP (2001) Multidrug resistance-like genes of Arabidopsis required for auxin transport and auxin-mediated development. Plant Cell 13:2441–2454

Nuhse TS, Stensballe A, Jensen ON, Peck SC (2004) Phosphoproteomics of the Arabidopsis plasma membrane and a new phosphorylation site database. Plant Cell 16:2394–2405

Odolczyk N, Fritsch J, Norez C, Servel N, da Cunha MF, Bitam S, Kupniewska A, Wiszniewski L, Colas J, Tarnowski K, Tondelier D, Roldan A, Saussereau EL, Melin-Heschel P, Wieczorek G, Lukacs GL, Dadlez M, Faure G, Herrmann H, Ollero M, Becq F, Zielenkiewicz P, Edelman A (2013) Discovery of novel potent DeltaF508-CFTR correctors that target the nucleotide binding domain. EMBO Mol Med 5:1484–1501

Park J, Song WY, Ko D, Eom Y, Hansen TH, Schiller M, Lee TG, Martinoia E, Lee Y (2012) The phytochelatin transporters AtABCC1 and AtABCC2 mediate tolerance to cadmium and mercury. Plant J 69:278–288

Paumi CM, Chuk M, Snider J, Stagljar I, Michaelis S (2009) ABC transporters in Saccharomyces cerevisiae and their interactors: new technology advances the biology of the ABCC (MRP) subfamily. Microbiol Mol Biol Rev 73:577–593

Paumi CM, Menendez J, Arnoldo A, Engels K, Iyer KR, Thaminy S, Georgiev O, Barral Y, Michaelis S, Stagljar I (2007) Mapping protein-protein interactions for the yeast ABC transporter Ycf1p by integrated split-ubiquitin membrane yeast two-hybrid analysis. Mol Cell 26: 15–25

Peck SC (2006) Phosphoproteomics in Arabidopsis: moving from empirical to predictive science. J Exp Bot 57:1523–1527

Peer WA, Murphy AS (2006) Flavonoids as signal molecules. Targets of flavonoid action. In: Grotewold E (ed) The science of flavonoids. Springer, Berlin, pp 239–268

Peer WA, Murphy AS (2007) Flavonoids and auxin transport: modulators or regulators? Trends Plant Sci 12:556–563

Peer WA, Brown DE, Tague BW, Muday GK, Taiz L, Murphy AS (2001) Flavonoid accumulation patterns of transparent testa mutants of arabidopsis. Plant Physiol 126:536–548

Peer WA, Bandyopadhyay A, Blakeslee JJ, Makam SN, Chen RJ, Masson PH, Murphy AS (2004) Variation in expression and protein localization of the PIN family of auxin efflux facilitator proteins in flavonoid mutants with altered auxin transport in Arabidopsis thaliana. Plant Cell 16:1898–1911

Peterson LB, Eskew JD, Vielhauer GA, Blagg BS (2012) The hERG channel is dependent upon the Hsp90alpha isoform for maturation and trafficking. Mol Pharm 9:1841–1846

Petrasek J, Cerna A, Schwarzerova K, Elckner M, Morris DA, Zazimalova E (2003) Do phytotropins inhibit auxin efflux by impairing vesicle traffic? Plant Physiol 131:254–263

Petrasek J, Mravec J, Bouchard R, Blakeslee JJ, Abas M, Seifertova D, Wisniewska J, Tadele Z, Kubes M, Covanova M, Dhonukshe P, Skupa P, Benkova E, Perry L, Krecek P, Lee OR, Fink GR, Geisler M, Murphy AS, Luschnig C, Zazimalova E, Friml J (2006) PIN proteins perform a rate-limiting function in cellular auxin efflux. Science 312:914–918

Quazi F, Molday RS (2011) Lipid transport by mammalian ABC proteins. Essays Biochem 50: 265–290

Radeva G, Perabo J, Sharom FJ (2005) P-Glycoprotein is localized in intermediate-density membrane microdomains distinct from classical lipid rafts and caveolar domains. FEBS J 272:4924–4937

Ranocha P, Dima O, Nagy R, Felten J, Corratge-Faillie C, Novak O, Morreel K, Lacombe B, Martinez Y, Pfrunder S, Jin X, Renou JP, Thibaud JB, Ljung K, Fischer U, Martinoia E, Boerjan W, Goffner D (2013) Arabidopsis WAT1 is a vacuolar auxin transport facilitator required for auxin homoeostasis. Nat Commun 4:2625

Rea PA (2007) Plant ATP-binding cassette transporters. Annu Rev Plant Biol 58:347–375

Rojas-Pierce M, Titapiwatanakun B, Sohn EJ, Fang F, Larive CK, Blakeslee J, Cheng Y, Cutler SR, Peer WA, Murphy AS, Raikhel NV (2007) Arabidopsis P-glycoprotein19 participates in the inhibition of gravitropism by gravacin. Chem Biol 14:1366–1376

Rumsby MG, Drew L, Warr JR (1998) Protein kinases and multidrug resistance. Cytotechnology 27:203–224

Ruzicka K, Strader LC, Bailly A, Yang H, Blakeslee J, Langowski L, Nejedla E, Fujita H, Itoh H, Syono K, Hejatko J, Gray WM, Martinoia E, Geisler M, Bartel B, Murphy AS, Friml J (2010) Arabidopsis PIS1 encodes the ABCG37 transporter of auxinic compounds including the auxin precursor indole-3-butyric acid. Proc Natl Acad Sci U S A 107:10749–10753

Sanchez-Fernandez R, Davies TGE, Coleman JOD, Rea PA (2001) The Arabidopsis thaliana ABC protein superfamily, a complete inventory. J Biol Chem 276:30231–30244

Santelia D, Henrichs S, Vincenzetti V, Sauer M, Bigler L, Klein M, Bailly A, Lee Y, Friml J, Geisler M, Martinoia E (2008) Flavonoids redirect PIN-mediated polar auxin fluxes during root gravitropic responses. J Biol Chem 283:31218–31226

Sauna ZE, Kim IW, Ambudkar SV (2007) Genomics and the mechanism of P-glycoprotein (ABCB1). J Bioenerg Biomembr 39:481–487

Scheidt HA, Vogel A, Eckhoff A, Koenig BW, Huster D (2007) Solid-state NMR characterization of the putative membrane anchor of TWD1 from Arabidopsis thaliana. Eur Biophys J 36: 393–404

Seino S, Takahashi H, Takahashi T, Shibasaki T (2012) Treating diabetes today: a matter of selectivity of sulphonylureas. Diabetes Obes Metab 14(Suppl 1):9–13

Shapiro AB, Ling V (1995) Reconstitution of drug transport by purified P-glycoprotein. J Biol Chem 270:16167–16175

Shirane M, Nakayama KI (2003) Inherent calcineurin inhibitor FKBP38 targets Bcl-2 to mitochondria and inhibits apoptosis. Nat Cell Biol 5:28–37

Sinko E, Ilias A, Ujhelly O, Homolya L, Scheffer GL, Bergen AA, Sarkadi B, Varadi A (2003) Subcellular localization and N-glycosylation of human ABCC6, expressed in MDCKII cells. Biochem Biophys Res Commun 308:263–269

Skach WR (2006) CFTR: new members join the fold. Cell 127:673–675

Snider J, Hanif A, Lee ME, Jin K, Yu AR, Graham C, Chuk M, Damjanovic D, Wierzbicka M, Tang P, Balderes D, Wong V, Jessulat M, Darowski KD, San Luis BJ, Shevelev I, Sturley SL, Boone C, Greenblatt JF, Zhang Z, Paumi CM, Babu M, Park HO, Michaelis S, Stagljar I (2013) Mapping the functional yeast ABC transporter interactome. Nat Chem Biol 9:565–572

Stolarczyk EI, Reiling CJ, Paumi CM (2011) Regulation of ABC transporter function via phosphorylation by protein kinases. Curr Pharm Biotechnol 12:621–635

Storch CH, Ehehalt R, Haefeli WE, Weiss J (2007) Localization of the human breast cancer resistance protein (BCRP/ABCG2) in lipid rafts/caveolae and modulation of its activity by cholesterol in vitro. J Pharmacol Exp Therap 323:257–264

Storer CL, Dickey CA, Galigniana MD, Rein T, Cox MB (2011) FKBP51 and FKBP52 in signaling and disease. Trend Endocrinol Metabol 22:481–490

Strader LC, Bartel B (2009) The Arabidopsis PLEIOTROPIC DRUG RESISTANCE8/ABCG36 ATP binding cassette transporter modulates sensitivity to the auxin precursor indole-3-butyric acid. Plant Cell 21:1992–2007

Strader LC, Bartel B (2011) Transport and metabolism of the endogenous auxin precursor indole-3-butyric acid. Mol Plant 4:477–486

Szabo K, Bakos E, Welker E, Muller M, Goodfellow HR, Higgins CF, Varadi A, Sarkadi B (1997) Phosphorylation site mutations in the human multidrug transporter modulate its drug-stimulated ATPase activity. J Biol Chem 272:23165–23171

Terasaka K, Blakeslee JJ, Titapiwatanakun B, Peer WA, Bandyopadhyay A, Makam SN, Lee OR, Richards EL, Murphy AS, Sato F, Yazaki K (2005) PGP4, an ATP binding cassette P-glycoprotein, catalyzes auxin transport in Arabidopsis thaliana roots. Plant Cell 17: 2922–2939

Theodoulou FL (2000) Plant ABC transporters. Biochimica Et Biophysica Acta-Biomembranes 1465:79–103

Timerman AP, Wiederrecht G, Marcy A, Fleischer S (1995) Characterization of an exchange reaction between soluble FKBP-12 and the FKBP.ryanodine receptor complex. Modulation by FKBP mutants deficient in peptidyl-prolyl isomerase activity. J Biol Chem 270:2451–2459

Titapiwatanakun B, Murphy AS (2009) Post-transcriptional regulation of auxin transport proteins: cellular trafficking, protein phosphorylation, protein maturation, ubiquitination, and membrane composition. J Exp Bot 60:1093–1107

Titapiwatanakun B, Blakeslee JJ, Bandyopadhyay A, Yang H, Mravec J, Sauer M, Cheng Y, Adamec J, Nagashima A, Geisler M, Sakai T, Friml J, Peer WA, Murphy AS (2009) ABCB19/PGP19 stabilises PIN1 in membrane microdomains in Arabidopsis. Plant J 57:27–44

Verrier PJ, Bird D, Burla B, Dassa E, Forestier C, Geisler M, Klein M, Kolukisaoglu U, Lee Y, Martinoia E, Murphy A, Rea PA, Samuels L, Schulz B, Spalding EJ, Yazaki K, Theodoulou FL (2008) Plant ABC proteins–a unified nomenclature and updated inventory. Trends Plant Sci 13: 151–159

Walker VE, Atanasiu R, Lam H, Shrier A (2007) Co-chaperone FKBP38 promotes HERG trafficking. J Biol Chem 282:23509–23516

Wang B, Henrichs S, Geisler M (2012) The AGC kinase, PINOID, blocks interactive ABCB/PIN auxin transport. Plant Signal Behav 7:1515–1517

Wang B, Bailly A, Zwiewka M, Henrichs S, Azzarello E, Mancuso S, Maeshima M, Friml J, Schulz A, Geisler M (2013) Arabidopsis TWISTED DWARF1 functionally interacts with auxin exporter ABCB1 on the root plasma membrane. Plant 25:202–214

Weiergraber OH, Eckhoff A, Granzin J (2006) Crystal structure of a plant immunophilin domain involved in regulation of MDR-type ABC transporters. FEBS Lett 580:251–255

Willemsen V, Friml J, Grebe M, van den Toorn A, Palme K, Scheres B (2003) Cell polarity and PIN protein positioning in Arabidopsis require STEROL METHYLTRANSFERASE1 function. Plant 15:612–625

Williams RJ, Spencer JPE, Rice-Evans C (2004) Flavonoids: antioxidants or signalling molecules? Free Rad Biol Med 36:838–849

Wu G, Otegui MS, Spalding EP (2010) The ER-localized TWD1 immunophilin is necessary for localization of multidrug resistance-like proteins required for polar auxin transport in Arabidopsis roots. Plant Cell 22:3295–3304

Yang H, Murphy AS (2009) Functional expression and characterization of Arabidopsis ABCB, AUX 1 and PIN auxin transporters in Schizosaccharomyces pombe. Plant J 59:179–191

Yang H, Richter GL, Wang X, Mlodzinska E, Carraro N, Ma G, Jenness M, Chao DY, Peer WA, Murphy AS (2013) Sterols and sphingolipids differentially function in trafficking of the Arabidopsis ABCB19 auxin transporter. Plant J 74:37–47

Yun UJ, Lee JH, Koo KH, Ye SK, Kim SY, Lee CH, Kim YN (2013) Lipid raft modulation by Rp1 reverses multidrug resistance via inactivating MDR-1 and Src inhibition. Biochem Pharmacol 85:1441–1453

Evolution of Transport Directionality in ABCBs

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Abstract Plant ATP-binding cassette subfamily B/P-glycoprotein/multidrug resistance (ABCB/PGP/MDR) proteins mediate the transport of a variety aliphatic and amphipathic substrates across the plasma membrane. An unexpected characteristic of some plant ABCBs that is not seen in animal homologs is uptake transport activity. However, in the best studied example of this phenomenon, the ABCB4 auxin transporter is associated with uptake only when intracellular auxin concentrations are low and exhibits canonical efflux activity when internal auxin concentrations increase. Physiological and biochemical characterizations of ABCB4 indicate that the protein serves as a homeostatic regulator and suggest evolutionary origins of the phenomenon. In this chapter we will review early transport studies and the discovery of putative uptake transporters in plants, the functional and structural evolution of these transporters, and what is known about the mechanisms of uptake and conditional uptake/efflux. Further, we will explore issues with homologous or heterologous unicellular systems and how these studies may have led to a mischaracterization of uptake transporters. A re-evaluation of current transport data and new ABCB mediated transport model will also be discussed.

1 Introduction

ATP-binding cassette (ABC) transporters form a ubiquitous super-family of proteins that utilize the energy from ATP hydrolysis to drive the transport of a variety of substrates across membrane bilayers. Found throughout the bacterial, animal and plant kingdoms, ABC transporters function in cellular detoxification (Martinoia

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et al. 2002), nutrient uptake (Singh and Rohm 2008), secretion of extracellular coating components (Qin et al. 2013), and transport of lipids and hormones (reviewed in Kang et al. 2011). The B-subclass of ABCs has been extensively characterized due to the association of P-glycoprotein (ABCB1/MDR1) and related ABCBs with polyspecific export of hydrophobic drug/xenobiotic efflux in animals. ABCB transporters can function either as full-length transporters or as homo- or hetero-dimers of transporters associated with antigen processing (TAPs) type half transporters (Chapter "Structure–Function of Plant ABC-Transporters"). All full-length ABCB transporters described to date are plasma membrane (PM) localized and are oriented with their 12 transmembrane helices (TMHs) spanning the PM and two nucleotide-binding domains (NBDs) extending into the cytosol. Solutions of crystal structures and functional analyses have shown that ABCB transporters can exist in either an inward/cytoplasmic facing or an outward/extracellular/apoplast facing state (Dawson and Locher 2007; Aller et al. 2009). An alternating access model of ABCB transport states that ATP hydrolysis causes conformation changes that shift transporters between these orientations, allowing for substrate binding and release across the PM (Zou and McHaourab 2009).

In plants, ABC transporters have been shown to function in the accumulation of secondary metabolites (Shitan et al. 2003, 2013; Stukkens et al. 2005), xenobiotic detoxification (Martinoia et al. 2002; Lee et al. 2005; Frelet-Barrand et al. 2008), transport of lipids to the cuticle (Pighin et al. 2004; Panikashvili et al. 2007; Luo et al. 2007), stomata aperture regulation (Lee et al. 2008), and transport of the phytohormones auxin (reviewed in Peer et al. 2011), abscisic acid (Kuromori et al. 2010), and cytokinin (Zhang et al. 2014). ABCB transporters were initially discovered in deduced amino acid sequence comparisons of mammalian ABCB1, under the premise that plant ABCBs would exhibit functions similar to mammalian ABCB1 in cellular detoxification (Dudler and Hertig 1992). However, subsequent biochemical studies have shown that plant ABCBs exhibit a much narrower substrate specificity compared to their mammalian homologs, and their roles in detoxification are minimal (reviewed in Conte and Loyd 2011). Instead, detoxification involving ABCs primarily involves ABCC/MRPs located at the tonoplast and a subset of ABCG/PDRs (Chapter "ABC Transporters and Heavy Metals").

The best characterized plant ABCBs are the Arabidopsis auxin efflux transporters ABCB1 and ABCB19, which mobilize the primary auxin indole-3-acetic acid (IAA) and contribute to the long distance polar transport of auxin from the shoot apex to the roots. ABCB1 is primarily localized apolarly in the shoot and root meristems (Sidler et al. 1998; Geisler et al. 2005; Mravec et al. 2009) and basally (top) in the cortex and endodermis in the boundary between the elongation and maturation zones of the root (Geisler et al. 2005). ABCB19 is primarily localized apolarly in the shoot apex, bundle sheath cells in shoots, cortical and stellar cells in the root, epidermal cells near the root apex, and also in cotyledons and anther filaments (Blakeslee et al. 2007; Wu et al. 2007; Lewis et al. 2009; Titapiwatanakun and Murphy 2009). *abcb1* and *abcb19* single mutants show ~25 % and >50 % reductions in rootward auxin transport (Noh et al. 2001; Blakeslee et al. 2007), and double mutants are reduced by ~70 % (Blakeslee et al. 2007). Phenotypes

consistent with this reduction in long distance rootward auxin transport include epinastic cotyledons, decreased inflorescence height, loss of apical dominance, increased lateral branching, reduced lateral root formation, and reduced stamen filaments (Noh et al. 2001, 2003; Geisler et al. 2005; Lewis et al. 2007).

Another well-characterized Arabidopsis ABCB auxin transporter is ABCB4, which exhibits efflux activity similar to ABCB1 and 19 at higher intracellular auxin concentrations, but mediates auxin uptake activity when internal auxin levels are very low (Terasaka et al. 2005; Yang and Murphy 2009; Kubeš et al. 2012). A highly similar paralog, ABCB21, appears to exhibit similar activities (Kamimoto et al. 2012). Plant ABCB uptake activity is not limited to auxin transporters, as alkaloid uptake has been associated with *Coptis japonica* ABCB1/MDR1 (Shitan et al. 2003), and Arabidopsis ABCB14 has been shown to function in malate/citrate uptake (Lee et al. 2008). This chapter will review the discoveries of plant ABCB transporters with uptake activity, their apparent functional divergence from exporters, structural features that are unique in putative uptake transporters, and the currently accepted mechanism of uptake and conditional transport directionality. Additionally, uptake transporter data will be re-evaluated and a new model of controlled ABCB mediated efflux will be discussed.

2 Discovery of Plant ABCB Uptake Transporters

***Coptis japonica* ABCB1** The first reported putative ABCB uptake transporter was ABCB1/MDR1 from *Coptis japonica* (Shitan et al. 2003). Berberine, an alkaloid with strong antimicrobial properties and medicinal uses, is synthesized in the *C. japonica* root and accumulates in the rhizomes. In cultured *C. japonica* cells and xenopus oocytes expressing CjABCB1, endogenous and exogenous berberine were shown to be actively taken up by cells via a mechanism that is ATP-dependent and sensitive to inhibitors of mammalian ABCB1. Further, oocytes and *Saccharomyces cerevisiae* expressing CjABCB1 accumulated the berberine precursor reticuline and showed increased sensitivity to the mutagen 4-nitroquinoline N-oxide respectively. These results suggest that CjABCB1 mediates berberine uptake from the root into the rhizome and appears to exhibit a preference for berberine-like alkaloids. A study by the same group has recently shown that another ABCB isoform from *Coptis japonica* (CjABCB2) has nearly identical characteristics (Shitan et al. 2013).

***Arabidopsis thaliana* ABCB4** A second plant ABCB transporter with apparent uptake activity was subsequently described by several groups (Santelia et al. 2005; Terasaka et al. 2005). ABCB4 was reported to function primarily in the lateral root cap, root epidermis, and mature root cortical cells (Santelia et al. 2005; Terasaka et al. 2005). ABCB4 was reported to be apolarly localized in the root cap and basally (top) localized in the epidermis (Terasaka et al. 2005) Analysis of *abcb4* mutants showed light and sucrose-dependent root phenotypes. On 0.5–1 % sucrose

and under moderate light (100–120 uE), *abcb4* mutants exhibited reductions in primary root length and lateral root number (Terasaka et al. 2005). When grown under high light or on >1.5 % sucrose, *abcb4* mutants showed an increase in primary root length, lateral root number, and root hair length (Santelia et al. 2005; Terasaka et al. 2005). Auxin transport assays and free IAA quantitations revealed that *abcb4* mutants had reduced basipetal auxin movement from the root tip (Santelia et al. 2005; Terasaka et al. 2005). Rootward auxin transport from the shoot apex in *abcb4* mutants was not different from wild type; however, overexpression increased auxin transport (Terasaka et al. 2005). Expression of ABCB4 in *Saccharomyces cerevisiae* resulted in hypersensitivity to IAA and the toxic auxin analog 5-fluoroindole, suggesting a role in auxin uptake (Santelia et al. 2005). Consistent with this result, expression in mammalian HeLa cells resulted in a net increase in auxin accumulation and, surprisingly, treatment with NPA reverted apparent uptake to efflux (Terasaka et al. 2005). This reversion correlated to an increase in intracellular IAA and was the first report of substrate concentration-dependent regulation of ABCB activity.

A subsequent study of ABCB4 (Lewis et al. 2007) confirmed previous reports that *abcb4* mutant roots exhibit reduced shootward auxin transport, but no changes in rootward auxin transport activity. It was expected that this reduction in shootward auxin transport from the root tip would result in a reduction in gravitropic bending, as is the case in mutants of the auxin efflux carrier PIN2 (Chen et al. 1998). However, consistent with the earlier report from Terasaka et al. (2005), no reduction in gravitropic bending was observed and, instead, a small enhancement of gravitropic bending was reported. Visualization of DR5:GFP revealed auxin distributions in the *abcb4* root apex and elongation zone were less discrete than wild type.

The elongated root hair phenotype of *abcb4* mutants was further examined in Cho et al. (2007). This study showed that overexpression of *ABCB4* under the control of a root hair-specific promoter (*PE7*) caused a decrease in root hair elongation similar to what was observed with overexpression of PIN efflux carriers in root hairs. Further, overexpression of the AUX1 auxin uptake transporter enhanced root hair length. Although subsequent work has shown that *PE7* is not exclusive to root hairs (Kubeš et al. 2012), these results indicated that the primary function of ABCB4 in roots hairs is auxin efflux. The same study corroborated ABCB4-mediated efflux of the artificial auxin 1-naphtalene acetic acid (NAA) when ABCB4 was overexpressed in tobacco BY-2 cells (Cho et al. 2007).

Yang and Murphy (2009) provided a detailed characterization of the transport activity of ABCB4 expressed in *Schizosaccharomyces pombe*. *S. pombe* cells expressing ABCB4 showed initial IAA accumulation followed by IAA export, confirming the substrate-dependent switch to efflux observed in Terasaka et al. (2005). Doubling the amount of exogenous IAA doubled the initial amount of IAA accumulation and decreased the time for reversion to export. This result was consistent with reports of variability in *abcb4* phenotypes under conditions that alter auxin homeostasis and auxin transport (Gray et al. 1998; Geisler et al. 2005; Santelia et al. 2005; Terasaka et al. 2005; Cho et al. 2007; Lewis et al. 2007). This

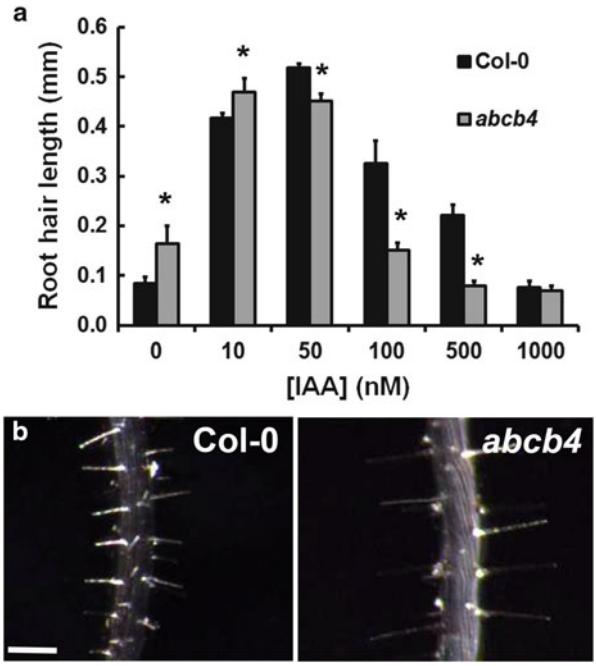

Fig. 1 ABCB4 regulates root hair elongation in Arabidopsis (5-day-old Arabidopsis seedlings). (**a**) Treatment with increasing indole-3-acetic-acid (IAA) concentrations inhibits root hair elongation. Mean lengths and SD from the first ten root hairs measured starting 1.5 mm from the root apex (ten seedling pools, $n = 3$). *$P < 0.05$. (**b**) Root hairs in a region 1.5 mm above theabcb4 root apex are consistently longer than in the wild type. Bar = 100 μm. (**c**) Visualization of ABCB4pro: ABCB4-GFP shows a signal at the plasma membrane in atrichoblast (a) and trichoblast (t) cells in light-grown seedlings acclimated to dim light. *Bar* = 10 μm. Figure taken from Kubeš et al. (2012)

same study also showed that 2,4-dichlorophenoxyacetic acid (2,4-D) inhibits ABCB4-mediated uptake activity. This observation raised questions about assays of ABCB4 conducted in BY-2, as BY-2 cells are regularly grown in media containing the synthetic auxin 2,4-D. The presence of 2,4-D in the assays reported by Cho et al. (2007) may have obscured uptake activity by elevating the intracellular auxin to levels where reversion to export would have already occurred.

In the most recent report of ABCB4, Kubeš et al. (2012) reported that auxin uptake into the root tip and that basipetal auxin transport in *abcb4* mutants is reduced, which is consistent with previous findings (Santelia et al. 2005; Terasaka et al. 2005; Lewis et al. 2007). Additionally, roots of *abcb4* mutants accumulate less auxin than wild type initially and switch to net accumulation after incubation with IAA. Consistent with Santelia et al. (2005) and Cho et al. (2007), root hairs in *abcb4* mutants are longer and addition of increasing exogenous auxin increases root hair length (Fig. 1). Further investigation of root hair phenotypes was not perused as expression of *ABCB4* under the root hair-specific promoter PE7 *and* auxin quantitation in root hairs were not successful. Expression in BY-2 suggests ABCB4

exhibits weak uptake activity. However, this result could not be investigated further, as lipophilic and AUX1/LAX-mediated 2,4-D uptake could not be inhibited, even after inhibition of AUX1 by 2-naphthoxyacetic acid (2-NOA). Expression of ABCB4 in *S. pombe* and HeLa cells produced similar concentration-dependent kinetics to those reported previously (Terasaka et al. 2005; Yang and Murphy 2009) and verified 2,4-D as a substrate for ABCB4 mediated uptake and inhibition of efflux activity.

From these combined results, it is evident that ABCB4 regulates cellular auxin levels primarily in the root epidermis by mediating auxin uptake when intracellular auxin levels are low and reverts to efflux when auxin concentration is increased.

***Arabidopsis thaliana* ABCB14 and ABCB21** Only two other ABCB transporters have been described to exhibit uptake activity. ABCB14 is expressed primarily in guard cells, where it regulates stomata closing in response to increased levels of CO_2 and exogenously applied malate (Lee et al. 2008). *ABCB14* expressed in *S. cerevisiae* and HeLa cells suggest that malate is taken up into the cells and that uptake activity is sensitive to the ABCB transporter inhibitors vanadate, verapamil, and cyclosporine A. Malate uptake was competed for by fumarate, and to a lesser extent succinate and citrate, suggesting a decrease in specificity compared to ABCB19. ABCB21, the closest homolog to ABCB4, is expressed on the abaxial side of leaves, in lateral organ junctions in shoots, and in root pericycle cells (Kamimoto et al. 2012). Expression in Arabidopsis protoplasts shows IAA and NAA export, though IAA appears to be the preferred substrate. ABCB21, like ABCB4, shows increased 5-FI sensitivity when expressed in *S. cerevisiae*. Assays in yeast expressing ABCB21 showed apparent IAA uptake when external IAA levels are high, and preloading with IAA returned IAA accumulation back down to control levels. Further, assays conducted with preloaded yeast cells and low external IAA showed net efflux activity. These results suggest that ABCB21 functions like ABCB4 in an intracellular auxin concentration-dependent manner.

3 Phylogeny of Putative Uptake Transporters and Functional Divergence

The Arabidopsis genome contains 21 full-length ABCB transporters. Phylogenetic analysis of these transporters reveals that they cluster into five clades (Fig. 2; Knöller et al. 2010). The auxin exporters ABCB1 and ABCB19 are found within clade II and are the only Arabidopsis transporters within this group. Clade I contains the malate/citrate uptake transporter ABCB14 along with ABCB13, ABCB2, and ABCB10. The function of the latter three transporters remains unknown; however, expression of ABCB2 in *S. pombe* suggests it is not a primary auxin transporter (Yang and Murphy 2009). Although grouped within the same clade, ABCB14 is clearly distinct from ABCB1 and ABCB19. No data for clade III or clade IV, which contain ABCB6, ABCB20, and ABCB15-18, have been

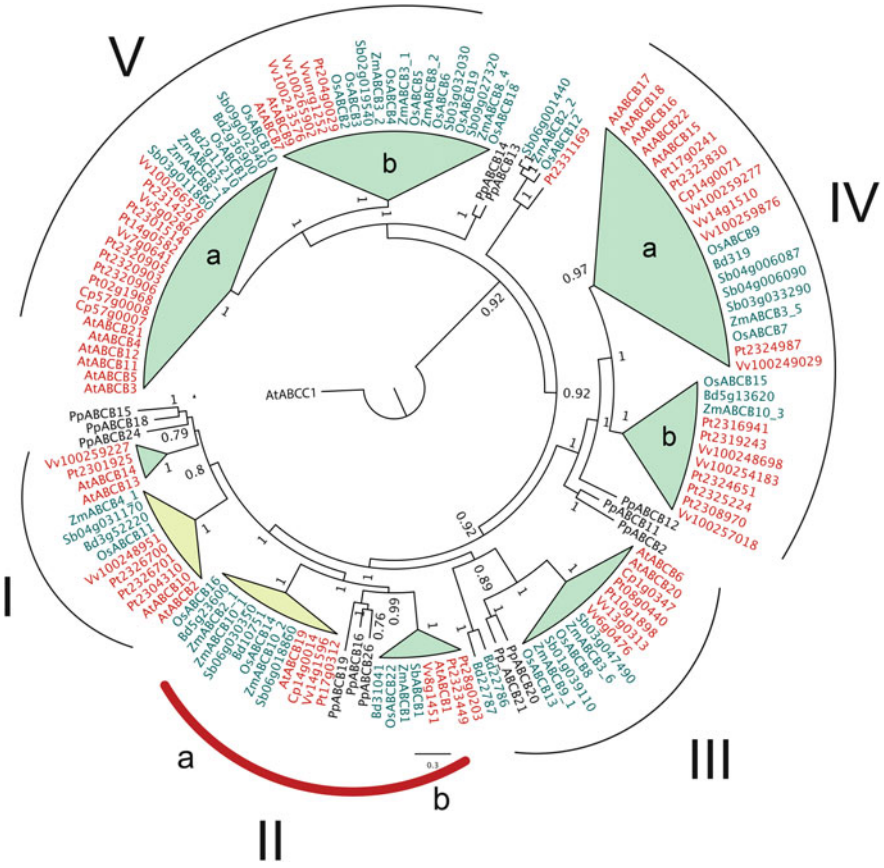

Fig. 2 Phylogenetic tree of plant ABCB transporters, including sequences from Arabidopsis, Vitis, Populus, Carica, Sorghum, Zea, Oryza, Brachypodium, and Physcomitrella. The phylogenetic tree can be divided into five clades (I–V). Branch numbers represent bootstrap values. Species names are colour coded for monocots (*green*) and dicots (*red*). Subclade Ib and IIa lie outside the Physcomitrella root (*yellow shaded subclades*). Figure taken from Knöller et al. (2010)

published to date. Preliminary studies of RNAi knockdowns of ABCB15-18 reveal that they may play roles in auxin transport and/or transport regulation (unpublished), but high sequence similarity of these genes due to duplication has made isolation and analysis of mutants difficult. The concentration dependent uptake/efflux auxin transporters ABCB4 and ABCB21 group together within clade V. The paralogous sets of ABCB3/5, ABCB11/12 and ABCB7/9 also group within clade V. ABCB11 has been shown to function in rootward auxin transport (Kaneda et al. 2011), however, the kinetics of ABCB11-mediated transport remain unknown. This data, along with biochemical and structure/function analyses, suggest a functional divergence of some ABCB transporters that cannot be predicted by sequence comparison alone (Knöller et al. 2010; Yang and Murphy 2009; Bailly et al. 2012).

4 Structural Features Unique to Putative Uptake Transporters

Crystal structures of a number of ABCB transporters have been elucidated, including *Staphylococcus aureus* Sav1866 and murine ABCB1 (Dawson and Locher 2007; Aller et al. 2009). These multidrug efflux transporters represent what are generally accepted as the resting states of efflux transporters (MmABCB1) and putative uptake transporters, or the substrate release state of exporters (Sav1866). No crystal structures have been determined for any plant ABCB transporter, however, homology modeling has provided valuable insight into unique structural features found in putative uptake transporters that are missing in the efflux specific transporters ABCB1 and ABCB19.

Structural models threaded on Sav1866 and MmABCB1 reveal that plant ABCB transporters share a common architecture (Yang and Murphy 2009; Bailly et al. 2012). Further, putative uptake ABCB transporters share better sequence alignments with crystal structures of exporters than prokaryotic importers, which suggest an exporter-like overall architecture. A predicted N-terminal coiled-coil domain is found in the structures of ABCB4, ABCB14, ABCB21, and CjABCB1, but not seen in the exporters ABCB1 or ABCB19. More detailed analysis of ABCB4 reveals that the hydrophobic region of transmembrane helix 4 (TMH4) is shifted inward, likely imbedding the apoplastic end of TM3 and TM4 within the PM. Additionally, this shift could change the distance between intracellular loop 2 (ILC2) and NBD2, which may rearrange the TMDs enough to alter transport directionality. Another coiled-coil domain is found in ABCB4 within the linker region connecting NBD1 and TMD2. This feature is not seen in the putative importers CjABCB1, ABCB14, and ABCB21 nor in the exporters ABCB1 or ABCB19. This suggests a specific role in regulating ABCB4 activity and not a general transport directionality mechanism. The function of these domains is unknown but may play a role in protein interactions and/or regulating transport properties. Obviously, the function of these features needs to be biochemically analyzed and is of utmost importance for understanding ABCB transporter function and regulation.

In addition to these structural features, analysis of substrate docking reveals kingdom-specific putative substrate binding sites within the TMDs. Docking of IAA to ABCB19, ABCB1, and ABCB4 in their outward facing states identified two putative substrate binding sites within the TMDs and associated with the inner leaflet of the PM (Yang and Murphy 2009; Bailly et al. 2012). ABCB4, however, had an additional binding site formed by TMH5 and 8 at the PM inner leaflet-cytosol interface not present in ABCB19. The proposed role of this additional site, which would explain the apparent "transport direction reversibility" seen with ABCB4 and ABCB21 will be discussed below.

5 Mechanism of Uptake and Conditional "Reversibility"

To date, no ABCB transporters with uptake activity have been reported in animals. Bacterial ABC transporters mediate the uptake of a number of primary metabolites, including maltose, vitamin B12, and histidine (reviewed in Linton and Higgins 2006). However, prokaryotic ABC uptake transporters are structurally and mechanistically dissimilar to eukaryotic transporters. In general, prokaryotic importers, such as the maltose importer $MalFGK_2$, have an associated periplasmic or cell-surface-associated substrate binding protein required for uptake activity (reviewed in George and Jones 2014). These binding proteins have high affinity and allow for substrate-specific and unidirectional transport.

Plant ABCB transporters probably do not have associated binding proteins and their specificities lie within the binding sites found within the TMDs. Substrate docking simulations of IAA with ABCB1, ABCB19 and ABCB4 reveal binding sites associated with the outer and inner leaflets of the PM. Detailed analyses of mammalian ABCB1 propose that a general hydrophobic-binding pocket associated primarily with the outer leaflet of the PM is responsible for the polyspecific exclusion of hydrophobic chemotherapy drugs. Similar binding positions are found in the exporters ABCB1 and ABCB19, however, surface electrostatic potentials at these positions differ (Bailly et al. 2012). This may explain the increased specificity in plant transporters compared to mammalian homologs. Docking simulations with outward facing models of ABCB4 and 14 also predict binding sites at these positions that may play roles in substrate binding and uptake. However, these sites have yet to be tested biochemically. Simulations have also identified two substrate binding sites associated with the inner leaflet of the PM for exporters and putative uptake transporters. These sites are not present in mammalian ABCB1 and likely also contribute to substrate specificity.

In addition to these sites the putative uptake transporter ABCB4 also contains a third inner-leaflet-associated binding site not found in exporters (Yang and Murphy 2009). This site is predicted to function as a regulatory site that can control the switch between apparent uptake and efflux activity. In this model, at low intracellular auxin concentrations, the regulatory binding site is unoccupied and ABCB4 is open to the apoplast. In this state ABCB4 has been predicted to mediate auxin uptake. Upon increased intracellular auxin levels, via putative transporter-mediated or endogenous uptake, and/or passive diffusion, the site is predicted to become occupied by auxin, inducing a conformation change in ABCB4 to inward/cytosol facing and allowing for export activity to occur. This concentration-dependent regulation or efflux activation appears to be unique to ABCB4, as ABCB1 and ABCB19 actually show increased efflux activity when auxin levels decrease (Geisler et al. 2005; Bouchard et al. 2006).

6 Re-Evaluation of the Data: Truly Reversible or Simply Control of Efflux?

While only a handful of ABCB transporters have been characterized to date, enough data has been produced to force us to ask a critical question: do ABCB transporters actually function in substrate uptake? While it is impossible to know for certain at this time a number of key concepts from ABCB4 suggest that this is not the case.

Increased free IAA levels in the root apex of *abcb4* mutants and apolar localization of ABCB4 in the root tip is consistent with a role in auxin export out of the root tip to the epidermis (Santelia et al. 2005; Terasaka et al. 2005). In gravitropism assays *abcb4* mutants show enhanced gravitropic bending and more diffuse auxin accumulations in the epidermis (Lewis et al. 2007), while *pin2* exhibits reduced bending and auxin accumulation at the root tip (Chen et al. 1998). This, along with the basal localization of ABCB4 in the elongation zone of the epidermis, (Terasaka et al. 2005) is consistent with a role in restricting auxin to the elongation zone until it is no longer needed for cell elongation. Basipetal auxin transport from the root tip is reduced in *abcb4* mutants (Terasaka et al. 2005; Lewis et al. 2007; Kubeš et al. 2012), also supporting a role in efflux. In trichoblast-forming epidermal cells, ABCB4 on the PM increases as the root hair elongates and decreases after elongation stops, and *abcb4* mutants exhibit longer root hairs than wild type (Kubeš et al. 2012). This suggests that ABCB4 is involved in efflux and removal of auxin from root hairs when root hair elongation is complete.

Studies in Arabidopsis roots reveal that uptake into the root tip is reduced in *abcb4* mutants (Santelia et al. 2005; Kubeš et al. 2012) and that overexpression is not different than wild type (Kubeš et al. 2012). Further, auxin uptake into the epidermis distal to the root tip, where auxin levels are decreased and ABCB4 would be expected to function as an uptake transporter, is not different in *abcb4* mutants (Santelia et al. 2005). These results suggest that the difference in apparent ABCB4-mediated uptake into the root apex is a consequence of an altered auxin gradient between the root tip and elongation zone.

Measuring transport directionality at the cellular level (i.e., uptake or efflux) *in planta* is not yet possible with modern techniques. Instead, transport assays using homologous or heterologous unicellular systems are performed. The issue here lies with what is actually measured in these systems: when apparent uptake is observed in assays, what is actually being measured is total cellular accumulation; a combination of endogenous and putative transporter mediated uptake, substrate embedded within the PM, and substrate bound to the transporter. In no case can we specifically separate uptake transport activity. If ABCB4 is initially open toward the apoplast, as current models suggest, then binding of substrates to the transporter and/or collection of extracellular substrate and partitioning into the PM without actual active substrate uptake may occur. This would support findings that putative uptake is less specific than export activity, and that uptake activity is low (Yang and Murphy 2009; Kubeš et al. 2012).

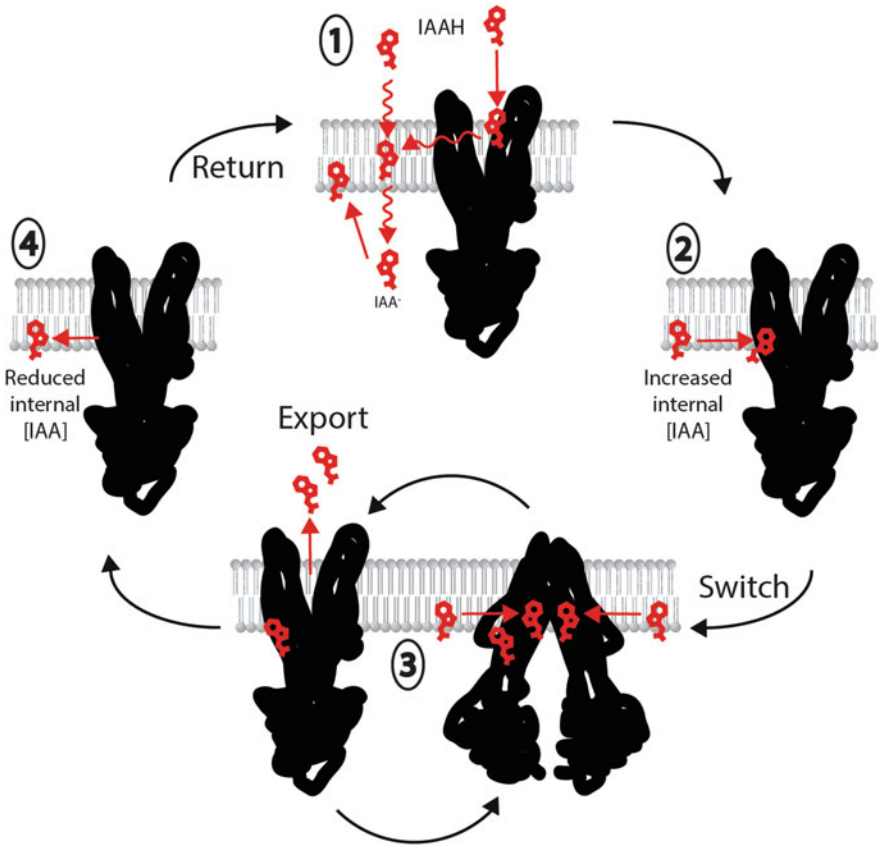

Fig. 3 Proposed model for ABCB4-mediated accumulation and activation of efflux. (1) ABCB4 is in its outward facing conformation when intracellular auxin concentrations are low. Protonated IAA can enter the cell by lipophilic diffusion or by binding to ABCB4, which collects IAA from the apoplast and accelerates diffusional uptake. (2) Once threshold intracellular auxin levels are reached, IAA binds to the regulatory binding site in ABCB4, (3) which causes a conformation change from outward/apoplast facing to inward/cytoplasm facing and allows export to occur. The export cycle continues until (4) intracellular auxin drops below the threshold level. When this occurs the regulatory site becomes unoccupied and ABCB4 returns to its outward facing "resting state" conformation

This suggests that, rather than ABCB4 being a conditionally reversible transporter, ABCB4 export activity is regulated by intracellular auxin concentrations (Fig. 3). In this model ABCB4 would remain in its outward facing "resting state" at low intracellular auxin concentrations. ABCB4 would not actively uptake auxin; however, auxin could accumulate within the cell via lipophilic diffusion and endogenous auxin transport. Collection of auxin from the apoplast by binding to ABCB4 exposed to the apoplast and partitioning into the PM may increase diffusion rates and cellular accumulation, hence the apparent uptake activity observed.

When threshold levels of intracellular auxin are reached, the third inner leaflet binding site becomes occupied and conformational changes in ABCB4 allow for export activity to begin. This controlled efflux would allow for the formation of auxin maxima and downstream auxin signaling events to occur and hold off export until auxin is no longer needed for physiological responses. Additionally, this regulation would also conserve energy, as ATP utilization would only occur when needed. This model likely applies to ABCB21 as well. Homologous and heterologous expression data show that ABCB4 and ABCB21 behave in a very similar manner. The lack of characterization in plants, however, makes the interpretation and physiological relevance of this data difficult to interpret.

While studies have only shown apparent uptake activity, it is suspected that CjABCB1 functions in a similar concentration-dependent manner as ABCB4. Studies characterizing berberine uptake activity in CjABCB1 has been conducted in *C. japonica* cell cultures, xenopus oocytes, and yeast (Shitan et al. 2003). Berberine, however, is toxic to *S. cerevisiae* and, like what is seen with ABCB4, this toxicity may obscure efflux activity. Assays of xenopus oocytes incubated in the presence of berberine or preloaded with berberine showed cells expressing CjABCB1 accumulated and retained more berberine than controls. In both of these cases levels of intracellular berberine may not have reached the threshold needed to activate export activity.

The model proposed for accumulation and controlled export of amphipathic substrates by Arabidopsis ABCB4 and ABCB21 may not be applicable to simple organic acids such as malate and citrate that are associated with Arabidopsis ABCB14 (Lee et al. 2008). Malate and citrate are highly soluble and not expected to interact with binding sites associated with the models of substrate accumulation proposed herein. Ongoing structural and mutational analyses are required to develop testable models of ABCB14 activity.

7 Conclusion

ABCB transporters with apparent uptake activity are, thus far, unique to the plant kingdom and serve vital roles in regulating plant growth and development. High sequence similarity and shared overall architecture throughout the ABCB protein family suggest a functional divergence from export-specific transporters. Several structural features are unique to these transporters and may function in regulating protein–protein interactions, substrate specificity, and transport activity. In particular, models identify a putative regulatory substrate-binding site that may alter transporter conformation and control the concentration dependent shift from apparent uptake to efflux.

Data suggests that some ABCB transporters have apparent uptake activity, however, experimental limitations of plant- and non-plant-based systems make this observation less than conclusive. The example provided by ABCB4 suggests that the uptake activity seen in homologous and heterologous unicellular systems

may be an increase in accumulation by transporter-assisted diffusion or simply substrate bound to the outward facing and overexpressed transporter. This suggests that, rather than having "reversible" uptake and efflux activity, ABCB4 export is regulated by intracellular auxin concentrations and may contribute to ATP-independent auxin accumulation. Characterization of additional transporters with differing substrates, as well as indepth structural modelling and mutational analyses are needed to provide a better understanding of the mechanisms and regulation of substrate transport in ABCB transporters.

References

Aller SG, Yu J, Ward A, Weng Y, Chittaboina S, Zhuo R, Harrell PM, Trinh YT, Zhang Q, Urbatsch IL, Chang G (2009) Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding. Science 323:1718–1722

Bailly A, Yang H, Martinoia E, Geisler M, Murphy AS (2012) Plant lessons: exploring ABCB functionality through structural modeling. Front Plant Sci 2:108

Blakeslee JJ, Bandyopadhyay A, Lee OR, Mravec J, Titapiwatanakun B, Sauer M, Makam SN, Cheng Y, Bouchard R, Adamec J, Geisler M, Nagashima A, Sakai T, Martinoia E, Friml J, Peer WA, Murphy AS (2007) Interactions among PIN-FORMED and P-glycoprotein auxin transporters in *Arabidopsis*. Plant Cell 19:131–147

Bouchard R, Bailly A, Blakeslee JJ, Oehring SC, Vincenzetti V, Lee OR, Paponov I, Palme K, Mancuso S, Murphy AS, Schulz B, Geisler M (2006) Immunophilin-like TWISTED DWARF1 modulates auxin efflux activities of Arabidopsis P-glycoproteins. J Biol Chem 281:30603–30612

Chen RJ, Hilson P, Sedbrook J, Rosen E, Caspar T, Masson PH (1998) The *Arabidopsis thaliana AGRAVITROPIC 1* gene encodes a component of the polar-auxin-transport efflux carrier. Proc Natl Acad Sci U S A 95:15112–15117

Cho M, Lee SH, Cho H (2007) P-glycoprotein4 displays auxin efflux transporter-like action in *Arabidopsis* root hair cells and tobacco cells. Plant Cell 19:3930–3943

Conte S, Loyd AM (2011) Exploring multiple drug and herbicide resistance in plants—Spotlight on transporter proteins. Plant Sci 180:196–203

Dawson RJP, Locher KP (2007) Structure of the multidrug ABC transporter sav1866 from *Staphylococcus aureusin* complex with AMP-PNP. FEBS Lett 581:935–938

Dudler R, Hertig C (1992) Structure of an mdr-like gene from *Arabidopsis thaliana* evolutionary implications. J Biol Chem 267:5882–5888

Frelet-Barrand A, Kolukisaoglu HU, Plaza S, Rüffer M, Azevedo L, Hörtensteiner S, Marinova K, Weder B, Schulz B, Klein M (2008) Comparative mutant analysis of Arabidopsis ABCC-Type ABC transporters: AtMRP2 contributes to detoxification, vacuolar organic anion transport and chlorophyll degradation. Plant Cell Physiol 49:557–569

Geisler M, Blakeslee JJ, Bouchard R, Lee OR, Vincenzetti V, Bandyopadhyay A, Titapiwa-tanakunB PWA, Bailly A, Richards EL, Ejendal KFK, Smith AP, Baroux C, Grossniklaus U, Müller A, Hrycyna CA, Dudler R, Murphy AS, Martinoia E (2005) Cellular efflux of auxin catalyzed by the *Arabidopsis* MDR/PGP transporter AtPGP1. Plant J 44:179–194

George M, Jones P (2014) Bacterial ABC transporters: structure and function. In: Remaut H, Fronzes R (eds) Bacterial membranes: structural and molecular biology. Caister Academic Press, Norfolk, VA

Gray WM, Östin A, Sandberg G, Romano CP, Estelle M (1998) High temperature promotes auxin-mediated hypocotyl elongation in Arabidopsis. Proc Natl Acad of Sci U S A 95:7197–7202

Kamimoto Y, Terasaka K, Hamamoto M, Takanashi K, Fukuda S, Shitan N, Sugiyama A, Suzuki H, Shibata D, Wang B, Pollmann S, Geisler M, Yazaki K (2012) Arabidopsis ABCB21 is a facultative auxin importer/exporter regulated by cytoplasmic auxin concentrations. Plant Cell Physiol 53:2090–2100

Kaneda M, Schuetz M, Lin BSP, Chanis C, Hamberger B, Western TL, Ehlting J, Samuels AL (2011) ABC transporters coordinately expressed during lignification of Arabidopsisstems include a set of ABCBs associated with auxin transport. J Exp Bot 62:2063–2077

Kang J, Park J, Choi H, Burla B, Kretzschmar T, Lee Y, Martinoia E (2011) Plant ABC transporters. Arabidopsis Book 9:e0153

Knöller AS, Blakeslee JJ, Richards EL, Peer WA, Murphy AS (2010) Brachytic2/*Zm*ABCB1 functions in IAA export from intercalary meristems. J Exp Bot 61:3689–3696

Kubeš M, Yang H, Richter GL, Cheng Y, Młodzińska E, Wang X, Blakeslee JJ, Carraro N, Petrášek J, Zažímalová E, Hoyerová K, Peer WA, Murphy AS (2012) The *Arabidopsis* concentration-dependent influx/efflux transporter ABCB4 regulates cellular auxin levels in the root epidermis. Plant J 69:640–654

Kuromori T, Miyaji T, Yabuuchi H, Shimizu H, Sugimoto E, Kamiya A, Moriyama Y, Shinozaki K (2010) ABC transporter AtABCG25 is involved in abscisic acid transport and responses. Proc Natl Acad Sci U S A 107:2361–2366

Lee M, Lee K, Lee J, Noh EW, Lee Y (2005) AtPDR12 contributes to lead resistance in Arabidopsis. Plant Physiol 138:827–836

Lee M, Choi Y, Burla B, Kim Y-Y, Jeon B, Maeshima M, Yoo J-Y, Martinoia E, Lee Y (2008) The ABC transporter AtABCB14 is a malate importer and modulates stomatal response to CO2. Nat Cell Biol 10:1217–1223

Lewis DR, Miller ND, Splitt BL, Wu GS, Spalding EP (2007) Separating the roles of acropetal and basipetal auxin transport on gravitropism with mutations in two *Arabidopsis Multidrug Resistance-Like* ABC transporter genes. Plant Cell 19:1838–1850

Lewis DR, Wu G, Lyung K, Spalding EP (2009) Auxin transport into cotyledons and cotyledon growth depend similarly on the ABCB19 Multidrug resistance-like transporter. Plant J 60:91–101

Linton KJ, Higgins CF (2006) Structure and function of ABC transporters: the ATP switch provides flexible control. Eur J Physiol 453:555–567

Luo B, Xue XY, Hu WL, Wang LJ, Chen XY (2007) An ABC transporter gene of *Arabidopsis thaliana*, AtWBC11, is involved in cuticle development and prevention of organ fusion. Plant Cell Physiol 48:1790–1802

Martinoia E, Klein M, Geisler M, Bovet L, Forestier C, Kolukisaoglu Ü, Muller-Röber B, Schulz B (2002) Multifunctionality of plant ABC transporters – more than just detoxifiers. Planta 214:345–355

Mravec J, Skůpa P, Bailly A, Hoyerová K, Krecek P, Bielach A, Petrásek J, Zhang J, Gaykova V, Stierhof Y-D, Dobrev PI, Schwarzerová K, Rolcík J, Seifertová D, Luschnig C, Benková E, Zazímalová E, Geisler M, Friml J (2009) Subcellular homeostasis of phytohormone auxin is mediated by the ER-localized PIN5 transporter. Nature 459:1136–1140

Noh B, Murphy AS, Spalding EP (2001) Multidrug resistance-like genes of Arabidops is required for auxin transport and auxin-mediated development. Plant Cell 13:2441–2454

Noh B, Bandyopadhyay A, Peer WA, Spalding EP, Murphy AS (2003) Enhanced gravi- and phototropism in plant *mdr* mutants mislocalizing the auxin efflux protein PIN1. Nature 423:999–1002

Panikashvili D, Savaldi-Goldstein S, Mandel T, Yifhar T, Franke RB, Höfer R, Schreiber L, Chory J, Aharoni A (2007) The Arabidopsis DESPERADO/AtWBC11 transporter is required for cutin and wax secretion. Plant Physiol 145:1345–1360

Peer WA, Blakeslee JJ, Yang H, Murphy AS (2011) Seven things we think we know about auxin transport. Mol Plant 4:487–504

Pighin JA, Zheng H, Balakshin LJ, Goodman IP, Western TL, Jetter R, Kunst L, Samuels AL (2004) Plant cuticular lipid export requires an ABC transporter. Science 306:702–704

Qin P, Tu B, Wang Y, Deng L, Quilichini TD, Li T, Wang H, Ma B, Li S (2013) ABCG15 encodes an ABC transporter protein, and is essential for post-meiotic anther and pollen exine development in Rice. Plant Cell Physiol 54:138–154

Santelia D, Vincenzetti V, Azzarello E, Bovet L, Fukao Y, Düchtig P, Mancuso S, Martinoia E, Geisler M (2005) MDR-like ABC transporter AtPGP4 is involved in auxin-mediated lateral root and root hair development. FEBS Lett 579:5399–5406

Shitan N, Bazin I, Dan K, Obata K, Kigawa K, Ueda K, Sato F, Forestier C, Yazaki K (2003) Involvement of CjMDR1, a plant multidrug-resistance-type ATP-binding cassette protein, in alkaloid transport in *Coptis japonica*. Proc Natl Acad Sci U S A 100:751–756

Shitan N, Dalmas F, Dan K, Kato N, Ueda K, Sato F, Forestier C, Yazaki K (2013) Characterization of Coptis japonica CjABCB2, an ATP-binding cassette protein involved in alkaloid transport. Phytochemistry 91:109–116

Sidler M, Hassa P, Hasan S, Ringli C, Dudler R (1998) Involvement of an ABC transporter in a developmental pathway regulating hypocotyl cell elongation in the light. Plant Cell 10:1623–1636

Singh B, Rohm KH (2008) Characterization of a *Pseudofdetoxmonas putid*a ABC transporter (AatJMQP) required for acidic amino acid uptake: biochemical properties and regulation by the Aau two-component system. Microbiology 154:797–809

Stukkens Y, Bultreys A, Grec S, Trombik T, Vanham D, Boutry M (2005) NpPDR1, a pleiotropic drug resistance-type ATP-binding cassette transporter from *Nicotiana plumbaginifolia*, plays a major role in plant pathogen defense. Plant Physiol 139:341–352

Terasaka K, Blakeslee JJ, Titapiwatanakun B, Peer WA, Bandyopadhyay A, Makam SN, Lee OR, Richards EL, Murphy AS, Sato F, Yazaki K (2005) PGP4, an ATP binding cassette P-glycoprotein, catalyzes auxin transport in *Arabidopsis thaliana* roots. Plant Cell 17:2922–2939

Titapiwatanakun B, Murphy AS (2009) Post-transcriptional regulation of auxin transport proteins: cellular trafficking, protein phosphorylation, protein maturation, ubiquitination, and membrane composition. J Exp Bot 60:1093–1107

Wu G, Lewis DR, Spalding EP (2007) Mutations in *Arabidopsis Multidrug Resistance-Like* ABC transporters separate the roles of acropetal and basipetal auxin transport in lateral root development. Plant Cell 19:1826–1837

Yang H, Murphy AS (2009) Functional expression and characterization of Arabidopsis ABCB, AUX 1 and PIN auxin transporters in Schizosaccharomyces pombe. Plant J 59:179–191

Zhang K, Novak O, Wei Z, Gou M, Zhang X, Yu Y, Yang H, Cai Y, Strnad M, Liu CJ (2014) *Arabidopsis* ABCG14 protein controls the acropetal translocation of root-synthesized cytokinins. Nat Commun 5. doi:10.1038/ncomms4274

Zou P, McHaourab HS (2009) Alternating access of the putative substrate-binding chamber in the ABC transporter MsbA. J Mol Biol 393:574–585

Trafficking of ABCB-type Auxin Transporters

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Abstract Trafficking of membrane proteins in plants is important for cellular homeostasis, cell–cell communication, and signaling. Identifying plant-specific endosomal components in exocytosis and endocytosis pathways has led to many advances in studies of plant membrane protein trafficking. The *Arabidopsis* ATP-binding cassette protein subfamily B (ABCB/PGP/MDR) family includes 21 genes and some of them mediate auxin distribution, but little is known about their intracellular trafficking. Among *Arabidopsis* ABCB proteins, only trafficking of ABCB1, ABCB4, and ABCB19 has been reported. ABCB1, ABCB4, and ABCB19 are localized symmetrically at the plasma membrane (PM) in most tissues and require TWISTED DWARF1 (TWD1) for PM targeting. In addition, the secretory pathway of ABCB19 to the PM is regulated by GNL1 at the Golgi which shows low abundance of ABCB19 in *gnl1*. ABCB4 and ABCB19 associate strongly in the PM and show non-dynamic and non-recycling features between the PM and early endosomes. ABCB4 slowly endocytoses to the vacuole via SNX1, and the pathway is GNOM independent but actin dependent. The role of ABCB19 and ABCB4 suggests that ABCB19 localization in the detergent resistant membrane contributes to PIN1 stabilization at the PM and that non-dynamic trafficking of ABCB4 may be involved in basal auxin homeostasis in cells.

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1 Introduction

Some plant membrane proteins are delivered to the sites of their action and retained at specific membrane domains and are occasionally recycled back or turned over by endomembrane trafficking mechanism upon internalization from the plasma membrane (PM). Trafficking of PM proteins in plants has been a recent focus, which explains the adaptability and flexibility of plants in response to various environmental changes to compensate their sessile lifestyle. Thus, membrane trafficking or the movement of membrane materials between the PM and endomembrane compartments is fundamentally necessary for cellular homeostasis, cell–cell communication, and external signal perception and transduction. In *Arabidopsis*, detailed intracellular trafficking studies of PM proteins have been performed with PIN-FORMED (PIN) auxin efflux carriers (mainly PIN1 and PIN2), the brassinosteroid receptor (BRI1), and the boron transporter (BOR1) (Geldner et al. 2003, 2007; Takano et al. 2010). However, the trafficking pathway of the ATP-binding cassette protein subfamily B proteins (ABABs) is barely understood. We summarize general information about the intracellular trafficking pathways in plant systems and then discuss the distinct trafficking pathway of ABCBs.

2 Membrane Trafficking

Membrane protein trafficking involves exocytosis/secretion and endocytosis. Biosynthesized membrane proteins in the endoplasmic reticulum (ER) and Golgi are delivered to the PM or to the lytic or storage vacuole and returned to the ER by sorting at the Golgi apparatus. Endocytosis is the process by which membrane proteins at the PM undergo constitutive recycling between the early endosome (EE) and the PM and traffic to the lytic vacuole for degradation or to the Golgi for sorting. The endomembrane system is conserved throughout eukaryotes and consists of the ER, the Golgi apparatus, endosomes and lytic compartments. In plants, the components of biosynthetic (secretory) and endocytic pathways are still not clearly defined. ER and Golgi stacks appear to behave as one dynamic system for the exchange of molecules (Brandizzi et al. 2002) without showing any intermediate compartments (Neumann et al. 2003). Plant endosomes are differently organized and there is also a unique part, which makes it difficult to apply knowledge from animal and yeast cells to plants. In animal and yeast cells, endosomes are divided into four classes based on their function: early, recycling, intermediate, and late endosomes. However, only early and late endosomes have been reported in plants. The typical tubule-vesicular early endosomes found in mammals have not been observed in plants, instead, the trans-Golgi network (TGN) is defined as an EE in which the endocytic marker FM4-64 reaches their first after a few minutes and serves as a major sorting station for the exocytic and endocytic pathways (Dettmer et al. 2006; Lam et al. 2007, 2009). EE is thought to be where PM proteins reach by

endocytosis and move back to the PM. VHA-a1 and Rab-A2/A3 GTPase are co-localized with TGN. The prevacuolar compartment (PVC) and multi vesicular compartment (MVC) are classified into late endosomes (LE) for sorting for degradation and retrieval to the TGN (Otegui and Spitzer 2008). Plant trafficking studies started by searching for homologs of animal and yeast endosome components. This misled to some components being located in the same endosomes where they are found in animal and yeast cells. For example, Rab GTPases regulate vesicle formation, transport, and fusion in early endosomes of animal cells (Zerial and McBride 2001). However, three Rab5-like proteins such as RHA1/AtRabF2a, ARA7/AtRabF2b, and ARA6/AtRabF1 are localized in LE in *Arabidopsis* (Kotzer et al. 2004; Haas et al. 2007). GNOM is one of the unique parts for plants. GNOM belongs to the Golgi BFA-resistant factor (GBF) family closed to GNOM LIKE 1 (GNL1) which is known for formation of COPI at the Golgi (Richter et al. 2007). GNOM appears to have ancestral function but is located in endosomes that merged with neither TGN nor LE, evolving extra for PIN recycling (Geldner et al. 2003).

3 The Exocytosis/Secretory Pathway

Exocytosis is the process by which proteins, lipids, and other molecules are delivered to the plasma membrane. This process usually starts from the ER, past the Golgi, TGN, and ends up with vesicle docking to and fusion with the PM. Fusion of the exocytic vesicle with the PM is part of the last step of exocytosis: vesicular cargo release and incorporation of membrane proteins and lipids into the membrane occurs. For proper targeting of newly synthesized proteins from the ER to the final destination requires vesicular trafficking and regulators such as RAB family proteins, ADP-ribosylation factor, RHO proteins of the Ras superfamly, and different type of soluble *N*-ethylmaleimide-sensitive factor adaptors (SNAREs; Jürgens 2004). Several types of vesicles including clathrin-coated vesicles (CCVs) and exocysts have been reported, and vesicle formation usually requires the adaptor protein (AP) complex to form CCVs, whereas vesicle fusion requires the SNARE complex.

4 Endocytosis

PM proteins in the endocytic pathway, endocytose from PM to EE and they move back to the PM for recycling and/or subsequently to the LE leading to the vacuole for degradation, in which TGN plays a role as a central sorting station for secretory, recycling, and degradation pathways. PVC/MVB/LE is a place for sorting PM proteins for degradation and mediates recycling vacuolar cargo receptors between LE and TGN. Following endocytosis, plasma membrane proteins, nutrient, lipids, peptide hormones, growth factors, and other extracellular molecules and fluids are

taken into cells. Thus, endocytosis is a crucial process for protein transport, membrane protein recycling, and cell signaling. Although several endocytic pathways (reviewed by Doherty and McMahon 2009) such as clathrin-mediated, caveolae/lipid raft-mediated, clathrin- and caveolae-independent fluid-phase endocytosis and phagocytosis are described in yeast and animals, knowledge in plants is very limited to only clathrin-mediated (Geldner et al. 2003) or clathrin-independent endocytosis (Samaj et al. 2004; Onelli et al. 2008).

Once internalized from the PM, the proteins are destined to recycle back to the PM or to the vacuole for degradation. Auxin-efflux carriers such as PIN1 and PIN2, brassinosteroid receptor (BRI1), and boron transporter (BOR1) appear constitutively following the recycling pathway (Geldner et al. 2003, 2007; Takano et al. 2010). The first constitutive endocytic recycling of PM proteins in plant was revealed for PIN1 via a pharmacological approach using the fungal macrocyclic lactone brefeldin A (BFA) (Steinmann et al. 1999; Geldner et al. 2001, 2003). BFA is an inhibitor of protein trafficking by preventing guanine-nucleotide exchange factors for the ADP-ribosylation factor GTPase (ARF-GEFs). BFA allows endocytosis from the PM but blocks flow back to the PM, which results in intracellular aggregation (BFA compartments). Thus, rapidly recycling proteins such as PINs, BRI1, and BOR1 accumulated in BFA-induced compartments. In case of PIN1, a molecular target of BFA is the ARF-GEF GNOM that is functionally associated with endosomes. Treatment of BFA trapped PIN1 with GNOM in BFA compartments by reducing PM localization (Geldner et al. 2003). This indicates that PIN1 undergoes constitutive recycling and the process is mediated by GNOM.

PIN2 endocytosis appears to be affected by GNL1, a BFA-insensitive ARF-GEF that has a conserved function in ER-to-Golgi trafficking. GNL1 is not involved in endosomal recycling pathway as GNOM is and there is no evidence that GNL1 and GNOM work in trafficking other PIN proteins. PIN2, BRI1, and an auxin influx carrier AUX1 are trafficking via a GNOM-independent endosomal trafficking regulator such as SYP61/VHA-a1 (Robert et al. 2008). The PIN2 protein distribution was dependent on SORTING NEXIN1 (SNX1), a component of the conserved retromer complex, which is sensitive to wortmannin, an inhibitor of phosphatidylinositol-3-kinase (PI3K) (Jaillais et al. 2006). PIN2 requires BFA-sensitive ARF-GEF other than GNOM for proper targeting of PIN2 into the lytic vacuole by gravity for degradation and PIN2 following recycling pathway via retromer components SNX1 and VACUOLAR PROTEIN SORTING29 (VPS29) from a PVC (Kleine-Vehn et al. 2008).

5 Intracellular Trafficking of Auxin-Transporting ABCB Proteins

5.1 Plant ABCBs

ABCB proteins are integral membrane proteins in which two transmembrane domains (TMD) span a pore inside the phospholipid bilayer. They also contain two cytosolic domains, the nucleotide-binding folds (NBF) that provide energy by binding and hydrolysis of ATP (Martinoia et al. 2002). ABCB proteins organize into two similar halves of TMD-NBF, which are connected by a linker domain (Geisler and Murphy 2006). Most studies on plant ABCB proteins have focused on determining their function but only five *Arabidopsis* ABCB proteins among 21 have had their function characterized (Cho and Cho 2013). Thus, investigations into their intracellular trafficking transporters are relatively less concerned.

The study of intracellular trafficking of ABCB proteins in *Arabidopsis* has been carried out with auxin transporters such as ABCB1 (PGP1), ABCB4 (PGP4/MDR4), and ABCB19 (PGP19/MDR1). In auxin biology, subcellular localization of PIN proteins changes dynamically in response to developmental and environmental cues. How do PIN proteins change their subcellular localization patterns? It is known that establishing a morphogen gradient by polar localized transporters and signal transduction from external cells is dependent on highly regulated intracellular trafficking. Furthermore, knowing that dynamic changes of PINs subcellular localization link with constitutive recycling between the PM and endosomes, plant intracellular trafficking of membrane proteins has been intensively studied with PINs.

ABCB proteins localize mainly in a non-polar manner in the PM but in some tissues polar localization is observed (Geisler et al. 2005; Terasaka et al. 2005; Blakeslee et al. 2007; Cho et al. 2007; Wu et al. 2007), although dynamic shifts in localization have not been demonstrated. ABCB1 proteins localize in the shoot and root apical meristem symmetrically, but polar localization is observed in mature cortical and endodermal cells in the distal elongation zone of the root (Geisler et al. 2005). The ABCB19 signal pattern at the PM in root seems to be tissue specific: more rootward signals in the epidermis, cortex and stele, and apolar signals in the pericycle, endodermis, and immature vascular cells (Blakeslee et al. 2007). However, GFP-ABCB19 expression is restricted to the stele and symmetrically localized according to signal observations with *ProABCB19:GFP-ABCB19*, whereas GFP-ABCB19 signals in cortical cells are symmetrical or nonsymmetrically distributed where the cells are located (Wu et al. 2007). ABCB4 is expressed in the root epidermis and lateral root cap (Terasaka et al. 2005). Although immunohistochemical analyses show polar localization in restricted root regions, the ABCB4-GFP signal is evenly distributed in the same region as well ABCB4 in other regions of the root (Cho et al. 2007).

Because ABCB proteins have auxin-transporting activity, people wondered how ABCB proteins cooperatively work with PIN proteins, although PIN proteins appear to be sufficient to generate auxin maximum. It was tested whether PIN subcellular localization and trafficking was affected in ABCBs loss of function mutants in an attempt to understand ABCB function during auxin transport. Additionally, ABCBs role in auxin transport is suggested through a comparative study of ABCB trafficking with that of PINs.

5.2 Regulation of the Secretory Pathway

Although plant secretory pathway is largely unknown, the roles of TWISTED DWARF1 (TWD1) and GNL1 in the ABCB transporter secretory pathway has been suggested (Titapiwatanakun et al. 2009; Wu et al. 2010; Wang et al. 2013) (Fig. 1).

TWD1 is a FKBP-type immunophilin containing multidomains such as a peptidyl-propyl isomerase (PPI)-like domain, tetratricopeptide repeat domain, a calmodulin domain, and a hydrophobic region (He et al. 2004; Geisler and Bailly 2007; see chapter "It Takes More Than Two to Tango: Regulation of Plant ABC Transporters"). TWD1 interacts with the cytosolic C terminus of ABCB1 and ABCB19 and is thought to be involved in protein folding and maturation through PPI activity and interaction with an HSP90 chaperone (Barik 2006). The C-terminal in-plane membrane anchored immunophilin (Bailly et al. 2013), TWD1 was co-purified with NPA-binding complexes when ABCB1, ABCB4, and ABCB19 were identified in high-affinity NPA binding fractions of DRM microdomains in *Arabidopsis* (Murphy et al. 2002). TWD1 was likely localized in the PM and is required for proper functioning of ABCB1 and ABCB19 auxin efflux activity (Bouchard et al. 2006; Geisler et al. 2003). However, a recent report described that TWD1 was not detected in the DRM and the existence of a GPI anchor in the protein could not be confirmed biochemically (Geisler et al. 2003; Titapiwatanakun et al. 2009; Wu et al. 2010). Moreover, ER localization of TWD1 has been demonstrated (Wu et al. 2010; Wang et al. 2013). Interestingly, ABCB1, ABCB4, and ABCB19 proteins were retained in the ER under a *twd1* background, and could not be delivered to the PM, whereas localization of PIN2 was not affected (Bouchard et al. 2006; Wu et al. 2010; Wang et al. 2013). In the *twd1* mutant, ABCB19 is not functional at the PM and shows reduction of polar auxin transport activity to 14 % of the wild type (Geisler et al. 2003). According to the fitting X-ray structure of TWD1, the nucleotide-binding domain (NBD) of the ABCB C terminus, which is conserved throughout all ABCB groups, is a possible site to interact (Granzin et al. 2006). Interestingly, PIN1 and PIN2 apparently are not delocalized in *twd1* (Bouchard et al. 2006; Wu et al. 2010). However, whether TWD1 specifically affects PM targeting of ABCB1, ABCB4, and ABCB19 or is also involved in the release of other ABCB proteins from the ER to the targeting organs remains an intriguing question.

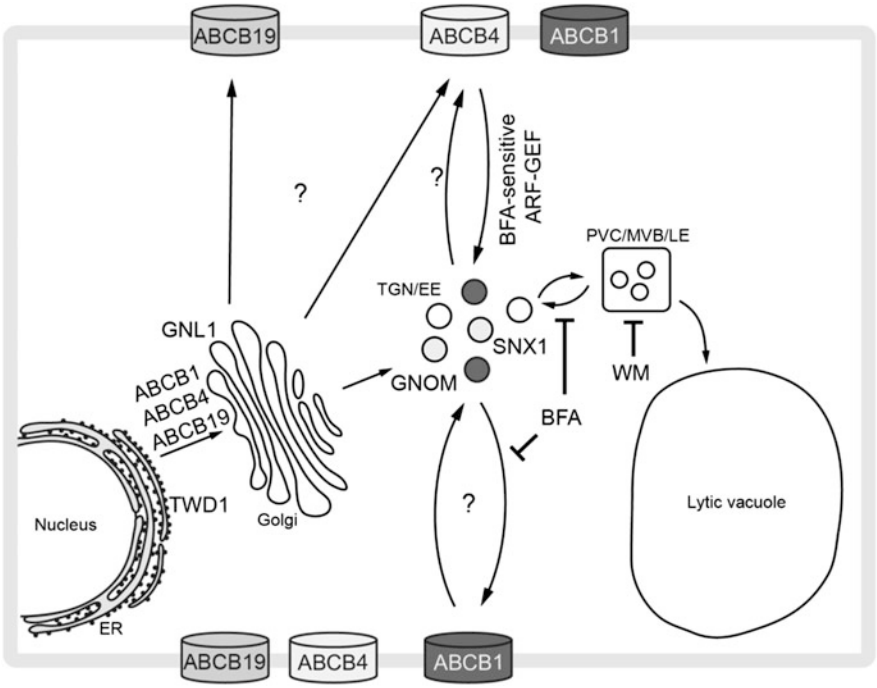

Fig. 1 Overview of intracellular trafficking of auxin-transporting ABCBs. *Arabidopsis* ABCB1, ABCB4, and ABCB19 among ABCBs are known as auxin transporters. They are evenly distributed at the PM in most tissues. TWD1 facilitates the release of ABCBs from ER and GNL1 regulates the secretion of ABCB19 to the PM. ABCB4 and ABCB19 are not constitutively recycled between the EE and the PM. ABCB4 endocytoses slowly to the vacuole via SNX1. Endocytosis of ABCB4 is mediated by the BFA-sensitive ARF-GEF. BFA inhibits recycling between the PM and EE as well as between EE and PVC/MVB/LE. WM interrupts vacuolar targeting. *ABCB* ATP binding cassette B, *ARF-GEF* guanine-nucleotide exchange factors for the ADP-ribosylation factor GTPase, *BFA* Brefeldin A, *EE* early endosome, *ER* endoplasmic reticulum, *GNL1* GNOM LIKE 1, *LE* late endosome, *MVB* multivesicular bodies, *PVC* pre-vacuolar compartments, *SNX1* SORTING NEXIN 1, *TGN* trans-Golgi network, *TWD1* TWISTED DWARF 1, *WM* wortmannin

GNL is an ARF-GEF that functions in COPI-coated vesicle formation and vesicle–cytoskeleton interactions in the secretory pathway (D'Souza-Schorey and Chavrier 2006; Geldner 2004). In *Arabidopsis*, only GBF and BIG families are known for ARF-GEFs. BFA-sensitive ARF-GEF, GNOM is the first characterized GBF but is required for endocytic recycling of PIN proteins from endosomes to the PM (Geldner et al. 2001). GNL1, the closest to GNOM, mainly performs the ancestral GBF function, although GNOM can substitute for the GNL1 function. GNL1 is a BFA-resistant ARF-GEF and is involved in the integrity of the Golgi and ER–Golgi trafficking in the secretory pathway by acting at the Golgi (Richter et al. 2007). The Golgi is usually intact in response to BFA, but in the absence of GNL1, the Golgi becomes sensitive to BFA and merges with the ER during BFA

treatment (Teh and Moore 2007). However, how this ancestral GBF function affects protein trafficking to the targeting organ has been hardly demonstrated. Instead, PIN2 endocytosis was selectively blocked in the *gnl1* mutant (Teh and Moore 2007). ABCB19 abundance on the PM was reduced in *gnl1* without changes in subcellular localization, suggesting that ABCB19 trafficking from the ER to Golgi where modification and maturation occur may be mediated by GNL1, which mainly regulates the abundance of ABCB19 at the PM (Titapiwatanakun et al. 2009).

5.3 ABCBs Endocytic Pathway

Studies about the ABCB protein endocytic pathway have focused on both recycling events and the degradation pathway (Fig. 1). Endocytic recycling is required for formation of polar localization, signal transduction, and reuse of PM proteins. For example, PIN proteins constitutively undergo endocytosis to the endosomes and are retargeted to the PM. This renders the PIN protein quickly transcytosed to other PM and changes the direction of the polar auxin maximum in response to developmental and environmental cues. BFA, a fungal toxin has been used as a chemical to test whether PM proteins undergo recycling. In *Arabidopsis*, BFA-sensitive ARF-GEF, GNOM unusually localizes in distinct endosomes, and BFA compartments contain TGN/EE including the GNOM-localized endosomes. BFA effects differ depending on tissue, time, and concentration, which vary in the contents of BFA compartments. In short-term BFA treatments (50 μM for 30 min) tested recycling of ABCB19 and ABCB1, ABCB19 did not form endosomal aggregation during short-term BFA treatment, whereas ABCB1 displays BFA compartments under the same condition (Titapiwatanakun et al. 2009). However, ABCB19 generated aggregations after 90 min treatment with 20 μM BFA (Wu et al. 2010; Wang et al. 2013) and ABCB4 formed BFA compartments at a low concentration (10 μM; Cho et al. 2007). ABCB4 aggregation in response to BFA in the background of a BFA-resistant $GNOM^{M696L}$–myc line showed that BFA sensitivity of ABCB4 trafficking is GNOM independent but requires other ARF-GEFs. Moreover, ABCB19 and ABCB4 sensitivities to BFA are different compared with that of PIN1 and PIN2. Namely, their PM abundance is less affected by BFA. BFA was thought to aggregate the endocytosed PM proteins that move to TGN, then to recycle to the PM whereas newly synthesized PM proteins are not significantly affected (Grebe et al. 2003). However, TGN is not only a recycling point for the PM but also for vacuolar-sorting receptors (VSRs) from the PVC to the TGN, which is included in BFA compartments (Niemes et al. 2010). Thus, whether formation of BFA compartments is accepted as an explanation for recycling should be carefully considered. Fluorescence recovery after photo bleaching (FRAP) analysis and tracing UV-induced green to red photoconvertible protein (Kaede) seem to be more reliable methods to test recycling of PM proteins (Ando et al. 2002; Sprague and McNally 2005). Fluorescence recovery time of ABCB4-GFP is proportional to the photobleached PM areas: ABCB4-GFP signals were recovered by lateral

diffusion rather than delivery from the intracellular pools. In addition, red-Kaede-ABCB4 in BFA-induced compartments did not appear in the PM after photoconversion and washout (Cho et al. 2012). FRAP analysis and ABCB4 fused with Kaede showed that a large portion of ABCB4 is stably anchored in the PM and does not undergo recycling.

Endocytosed ABCB4 merged with Sorting Nexin 1 (SNX1) that also colocalized with aggregation of ABCB4 after treatment with BFA. SNX1 was defined as a PVC/MVB/LE and colocalized with another LE marker RABF2b (ARA7) following treatment with wortmannin (Jaillais et al. 2008). However, the subcellular localization of SNX1 remains controversial. Several groups have reported that SNX1 localizes to the PVC (Jaillais et al. 2008; Kleine-Vehn et al. 2008), whereas another group showed partial colocalization of SNX1 with both PVC and TGN (Phan et al. 2008). Specific localization of SNX1 to TGN has been recently reported as the recycling point for VSRs from the PVC to the TGN (Niemes et al. 2010). PM proteins in LE destined for degradation. Dark treatment showed that ABCB4 traffics to the vacuole where degradation likely occurs. The meaning for the existence of ABCB4 in SNX1 needs to be addressed. However, ABCB4 seems to degrade via SNX1 to the vacuole but, interestingly, no other LE localized ARA7 and RHA1 merged with ABCB4. Wortmannin treatment, a PI-3 kinase inhibitor, blocks ABCB4 trafficking to the vacuole and results in dilated vesicles (Cho et al. 2012).

The prominent feature of ABCB4 trafficking is a slow and nondynamic trafficking to the vacuole, which is consistent with nonpolar and stationary PM localization of proteins. Only a small portion of ABCB4s appear to be involved in endocytic degradation pathway and most are retained in the PM. ABCB19 apparently has similar characteristics. ABCB1, ABCB4, and ABCB19 found in the DRM of the PM where C18 fatty acids, glucosylceramide, and sitosterol are enriched (Titapiwatanakun et al. 2009). In the MDCK cell line, glycosylphosphatidylinositol (GPI)-anchored proteins and transmembrane proteins that associate with DRM are preferentially targeted to the apical side (Brown and London 1998; Simons and Ikonen 1997). In addition, PGP/ABCB and GPI-anchored proteins play a role in DRM maintenance important for formation of multiprotein complexes in mammals (Borner et al. 2005). Although some observation of asymmetrical localization of ABCBs might be related to DRM localization, ABCB19 in DRMs seems to be crucial for PIN trafficking and proper auxin transport. ABCB19 is solely detected in DRM and PIN1 localizes both in DRM and Triton-X100-soluble fractions. Therefore, ABCB19 seems to affect PIN1 trafficking by maintaining the DRM and/or by the interaction with PIN1 in the DRM. In the absence of ABCB19, PIN1 trafficking was disturbed. For example, some PIN1 proteins after BFA washout are not restored to the PM. As a result, ABCB19 regulates the auxin stream by interrupting PIN stability in the DRM (Titapiwatanakun et al. 2009). This may result in more severely defected shoot basipetal IAA transport in *abcb19* and *abcb19*/*abcb1* than in *pin1-7* (Blakeslee et al. 2007). The role of stable and persistent residence of ABCB4 in the PM was hypothesized that ABCB4 may be appropriate for basal auxin transport to maintain the homeostatic cellular auxin level, whereas dynamic

intracellular trafficking of PINs regulates directional auxin transport in response to internal and environmental cues. For example, PIN2 is localized shootward in root epidermal cells facilitating basipetal auxin transport and root gravitropism (Blilou et al. 2005). In contrast, ABCB4 is expressed symmetrically in the root epidermis and may supply auxin for the epidermal cells to maintain the basal auxin concentration necessary for their growth and other auxin-mediated fundamental events.

5.4 The Role of the Cytoskeleton in ABCB Trafficking

The cytoskeleton is involved in intracellular trafficking of membrane proteins. Actin and microtubules provide a framework for cells as well as a road network for membrane protein trafficking (Apodaca 2001). PIN cycling is dependent on actin but is independent of microtubules. Treatment of actin with a depolymerizing agent induces some intracellular dots without changes in polar PIN localization and inhibits the BFA effect on PIN cycling (Geldner et al. 2001).

ABCB19 localization is not strikingly affected by the actin inhibitor, latrunculin B, or the microtubule inhibitor, oryzalin (Titapiwatanakun et al. 2009). However, actin is involved in ABCB4 endocytosis, as well as targeting of ABCB4 to the PM. But intracellular aggregation of ABCB4 after treatment with cytochalasin D is less sensitive than for PINs, which might be due to a less dynamic ABCB4 trafficking (Cho et al. 2012). TIBA (2,3,5-triiodobenzoic acid), a weak auxin and auxin transport inhibitor, also inhibits endocytosis and vesicle motility by interfering with actin stabilization (Dhonukshe et al. 2008). TIBA does not change localization of ABCB4 but it interrupts the action of BFA on ABCB4. Microtubules did not significantly affect ABCB4 trafficking as shown with the microtubule-disrupting agent oryzalin (Cho et al. 2012).

6 Trafficking of Mammalian MDR/PGPs

The trafficking pathways of *Arabidopsis* ABCB orthologs have slightly different targeting routes along cell types but their association with the endosomal reservoir to regulate abundance in common. Newly synthesized rat MDR1 and MDR2 in hepatocytes move rapidly via the Golgi directly to the apical bile canalicular membrane (Kipp and Arias 2000) compared with another ABC transporter, sister of p-glycoprotein (SPGP), targeting the bile canaliculus through endosomal compartments after release from the Golgi (Kipp et al. 2001). The abundance of MDR at the bile canaliculus increases due to second messenger AMP and the bile salt taurocholate even though protein synthesis is inhibited. This observation suggests that intracellular pools exist between the bile canalicular membrane and lysosome in the degradation pathway (Kipp et al. 2001) and that MDR cycles between them after reaching the bile canaliculus. ABCA1 and human MDR1/P-gp are also

associated with the endosomal compartment and undergo recycling with the PM (Santamarina-Fojo et al. 2001; Kim et al. 1997). Interestingly, when functional P-gp-EGFP is stably transfected into human breast cancer MCF-7 cells, newly synthesized P-gp-EGFP is delivered to the PM via early endosomes. Subsequently, P-gp-EGFP at the PM moves to the early endosomes and to the lysosome for the degradation (Fu and Roufogalis 2007).

cAMP-induced MDR trafficking is analogous to the insulin-responsive glucose transporter 4 (GLUT4), which moves from intracellular pool to the PM in response to cAMP and insulin (Pessin et al. 1999). PI3K also regulates recruitment of the ABC transporter from the intracellular pool to the bile canalicular membrane after activation by taurocholate as well as ABC transport function. Treatment with the PI3K inhibitor, wortmannin prior to taurocholate, prevents trafficking from the intracellular pool to the bile canalicular membrane and reduces bile secretion (Misra et al. 1999). Intracellular trafficking of P-gp-EGFP is actin dependent and less affected by microtubules. Applying the actin depolymerizing agents, cytochalasin D and latrunculin B, causes intracellular retention of P-gp-EGFP (Fu and Roufogalis 2007).

7 Conclusion and Future Prospects

Plant ABCB trafficking has been primarily investigated for *Arabidopsis* PM auxin transporters, ABCB1, ABCB4, and ABCB19. Identification of plant endosomal components and their different organization shows that the plant trafficking pathways are partially distinct from that of mammalian ABCBs. Although mammalian ABCB proteins undergo recycling, *Arabidopsis* ABCB19 and ABCB4 retain widely on the PM and ABCB4 endocytoses slowly to the vacuole. The trafficking pathway of these proteins was investigated to understand the role of ABCB proteins in auxin transport and particularly their relationship with PINs. While polar localization of PIN proteins and the dynamic changes are linked to recycling events, ABCB proteins localize in a nonpolar manner and their trafficking is less dynamic compared with that of PINs. Although the contribution of ABCB proteins to PIN stabilization and basal auxin homeostasis in cells has been suggested, how they work together remains to be elucidated in detail.

In this chapter, we focused on three ABCB auxin transporters localized in the PM. However, some other ABCB proteins localize to the membrane of diverse cellular organs such as vacuoles, chloroplasts, or mitochondria (Kang et al. 2011) and we are unaware of the functions and subcellular localization of many other ABCB proteins. Studies of intracellular trafficking provide insight into how ABCB proteins move to the target membrane and endocytose for degradation and how both trafficking pathways are regulated. This information is helpful to develop a strategy to modulate spatial and temporal distribution of ABCB proteins, which is important to control their functions for further applications.

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References

Ando R, Hama H, Yamamoto-Hino M et al (2002) An optical marker based on the UV-induced green-to-red photoconversion of a fluorescent protein. Proc Natl Acad Sci U S A 99:12651–12656

Apodaca G (2001) Endocytic traffic in polarized epithelial cells: role of the actin and microtubule cytoskeleton. Traffic 2:149–159

Bailly A, Wang B, Zwiewka M et al (2013) Expression of TWISTED DWARF1 lacking its in-plane membrane anchor leads to increased cell elongation and hypermorphic growth. Plant J 77:108–118

Barik S (2006) Immunophilins: for the love of proteins. Cell Mol Life Sci 63:2889–2900

Blakeslee JJ, Bandyopadhyay A, Lee OR et al (2007) Interactions among PINFORMED (PIN) and P-glycoprotein (PGP) auxin transporters in *Arabidopsis thaliana*. Plant Cell 19:131–147

Blilou I, Xu J, Wildwater M et al (2005) The PIN auxin efflux facilitator network controls growth and patterning in *Arabidopsis* roots. Nature 433:39–44

Borner GH, Sherrier DJ, Weimar T et al (2005) Analysis of detergent-resistant membranes in *Arabidopsis*. Evidence for plasma membrane lipid rafts. Plant Physiol 137:104–116

Bouchard R, Bailly A, Blakeslee JJ et al (2006) Immunophilin-like TWISTED DWARF1 modulates auxin efflux activities of *Arabidopsis* P-glycoproteins. J Biol Chem 281:30603–30612

Brandizzi F, Frangne J, Marc-Martin S (2002) The destination for single-pass membrane proteins is influenced markedly by the length of the hydrophobic domain. Plant Cell 14:1077–1092

Brown DA, London E (1998) Functions of lipid rafts in biological membranes. Annu Rev Cell Dev Biol 14:111–136

Cho M, Cho H-T (2013) The function of ABCB transporters in auxin transport. Plant Signal Behav 8:e22990

Cho M, Lee SH, Cho H-T (2007) P-glycoprotein4 displays auxin efflux transporter-like action in *Arabidopsis* root hair cells and tobacco cells. Plant Cell 19:3930–3943

Cho M, Lee ZW, Cho H-T (2012) ATP-binding cassette B4, an auxin-efflux transporter, stably associates with the plasma membrane and shows distinctive intracellular trafficking from that of PIN-FORMED proteins. Plant Physiol 159:642–654

Dettmer J, Hong-Hermesdorf A, Stierhof YD et al (2006) Vacuolar H^+-ATPase activity is required for endocytic and secretory trafficking. Plant Cell 18:715–730

Dhonukshe P, Grigoriev I, Fischer R et al (2008) Auxin transport inhibitors impair vesicle motility and actin cytoskeleton dynamics in diverse eukaryotes. Proc Natl Acad Sci U S A 105:4489–4494

Doherty GJ, McMahon HT (2009) Mechanisms of endocytosis. Annu Rev Biochem 78:857–902

D'Souza-Schorey C, Chavrier P (2006) ARF proteins: roles in membrane traffic and beyond. Nat Rev Mol Cell Biol 7:347–358

Fu D, Roufogalis BD (2007) Actin disruption inhibits endosomal traffic of P-glycoprotein-EGFP and resistance to daunorubicin accumulation. Am J Physiol 292:C1543–C1552

Geisler M, Bailly A (2007) Tête-à-tête: the function of FKBPs in plant development. Trends Plant Sci 12:465–473

Geisler M, Murphy AS (2006) The ABC of auxin transport: the role of p-glycoproteins in plant development. FEBS Lett 580:1094–1102

Geisler M, Kolukisaoglu HU, Bouchard R et al (2003) TWISTED DWARF1, a unique plasma membrane-anchored immunophilin-like protein, interacts with *Arabidopsis* multidrug resistance-like transporters AtPGP1 and AtPGP19. Mol Biol Cell 14:4238–4249

Geisler M, Blakeslee JJ, Bouchard R et al (2005) Cellular efflux of auxin catalyzed by the *Arabidopsis* MDR/PGP transporter AtPGP1. Plant J 44:179–194

Geldner N (2004) The plant endosomal system–its structure and role in signal transduction and plant development. Planta 219:547–560

Geldner N, Friml J, Stierhof YD et al (2001) Auxin transport inhibitors block PIN1 cycling and vesicle trafficking. Nature 413:425–428

Geldner N, Anders N, Wolters H et al (2003) The *Arabidopsis* GNOM ARF-GEF mediates endosomal recycling, auxin transport, and auxin-dependent plant growth. Cell 112:219–230

Geldner N, Hyman D, Wang X et al (2007) Endosomal signaling of plant steroid receptor kinase BRI1. Genes Dev 21:1598–1602

Granzin J, Eckhoff A, Weiergräber OH (2006) Crystal structure of a multi-domain immunophilin from *Arabidopsis thaliana*: a paradigm for regulation of plant ABC transporters. J Mol Biol 364:799–809

Grebe M, Xu J, Möbius W et al (2003) *Arabidopsis* sterol endocytosis involves actinmediated trafficking via ARA6-positive early endosomes. Curr Biol 13:1378–1387

Haas TJ, Sliwinski MK, Martínez DE et al (2007) The *Arabidopsis* AAA ATPase SKD1 is involved in multivesicular endosome function and interacts with its positive regulator LYST-INTERACTING PROTEIN5. Plant Cell 19:1295–1312

He Z, Li L, Luan S (2004) Immunophilins and parvulins. Superfamily of peptidyl prolyl isomerases in *Arabidopsis*. Plant Physiol 134:1248–1267

Jaillais Y, Fobis-Loisy I, Miege C et al (2006) AtSNX1 defines an endosome for auxin-carrier trafficking in *Arabidopsis*. Nature 443:106–109

Jaillais Y, Fobis-Loisy I, Miège C et al (2008) Evidence for a sorting endosome in *Arabidopsis* root cells. Plant J 53:237–247

Jürgens G (2004) Membrane trafficking in plants. Annu Rev Cell Dev Biol 20:481–504

Kang J, Park J, Choi H et al. (2011) Plant ABC transporters. Arabidopsis Book 9:e0153. doi: 10.1199/tab.0153

Kim H, Barroso M, Samanta R et al (1997) Experimentally induced changes in the endocytic traffic of P-glycoprotein alter drug resistance of cancer cells. Am J Physiol 273:C687–C702

Kipp H, Arias IM (2000) Newly synthesized canalicular ABC transporters are directly targeted from the Golgi to the hepatocyte apical domain in rat liver. J Biol Chem 275:15917–15925

Kipp H, Pichetshote N, Arias IM (2001) Transporters on demand: intrahepatic pools of canalicular ATP binding cassetted transporters in rat liver. J Biol Chem 276:7218–7224

Kleine-Vehn J, Leitner J, Zwiewka M et al (2008) Differential degradation of PIN2 auxin efflux carrier by retromer-dependent vacuolar targeting. Proc Natl Acad U S A 105:17812–17817

Kotzer AM, Brandizzi F, Neumann U et al (2004) AtRabF2b (Ara7) acts on the vacuolar trafficking pathway in tobacco leaf epidermal cells. J Cell Sci 117:6377–6389

Lam SK, Tse YC, Robinson DG (2007) Tracking down the elusive early endosome. Trends Plant Sci 12:497–505

Lam SK, Cai Y, Tse YC et al (2009) BFA-induced compartments from the Golgi apparatus and trans-Golgi network/early endosome are distinct in plant cells. Plant J 60:865–881

Martinoia E, Klein M, Geisler M et al (2002) Multifunctionality of plant ABC transporters-more than just detoxifiers. Planta 214:345–355

Misra S, Ujházy P, Varticovski L (1999) Phosphoinositide 3-kinase lipid products regulate ATP-dependent transport by sister of P-glycoprotein and multidrug resistance associated protein 2 in bile canalicular membrane vesicles. Proc Natl Acad Sci U S A 96:5814–5819

Murphy AS, Hoogner KR, Peer WA et al (2002) Identification, purification, and molecular cloning of N-1-Naphthylphthalmic acid-binding plasma membrane-associated Aminopeptidases from *Arabidopsis*. Plant Physiol 128:935–950

Neumann U, Brandizzi F, Hawes C (2003) Protein transport in plant cells: in and out of the Golgi. Ann Bot 92:167–180
Niemes S, Langhans M, Viotti C et al (2010) Retromer recycles vacuolar sorting receptors from the trans-Golgi network. Plant J 61:107–121
Onelli E, Prescianotto-Baschong C, Caccianiga M et al (2008) Clathtin-dependent and independent endocytic pathways in tobacco protoplasts revealed by labelling with charged nanogold. J Exp Bot 59:3051–3069
Otegui MS, Spitzer C (2008) Endosomal functions in plants. Traffic 9:1589–1598
Pessin JE, Thurmond D, Elmendorf JS et al (1999) Molecular basis of insulin-stimulated GLUT4 vesicle trafficking. LOCATION! LOCATION! LOCATION! J Biol Chem 274:2593–2596
Phan NQ, Kim SJ, Bassham DC (2008) Overexpression of *Arabidopsis* sorting nexin AtSNX2b inhibits endocytic trafficking to the vacuole. Mol Plant 1:961–976
Richter S, Geldner N, Schrader J et al (2007) Functional diversification of closely related ARF-GEFs in protein secretion and recycling. Nature 448:488–492
Robert S, Chary SN, Drakakaki G et al (2008) Endosidin 1 defines a compartment involved in endocytosis of the brassinosteroid receptor BRI1 and the auxin transporters PIN2 and AUX1. Proc Natl Acad U S A 105:8464–8469
Samaj J, Baluska F, Voigt B et al (2004) Endocytosis, actin cytoskeleton, and signaling. Plant Physiol 135:1150–1161
Santamarina-Fojo S, Remaley AT, Neufeld EB et al (2001) Regulation and intracellular trafficking of the ABCA1 transporter. J Lipid Res 42:1339–1345
Simons K, Ikonen E (1997) Functional rafts in cell membranes. Nature 387:569–572
Sprague BL, McNally JG (2005) FRAP analysis of binding, proper and fitting. Trends Cell Biol 15:84–91
Steinmann T, Geldner N, Grebe M et al (1999) Coordinated polar localization of auxin efflux carrier PIN1 by GMON ARF GEF. Science 286:316–318
Takano J, Tanaka M, Toyoda A et al (2010) Polar localization and degradation of *Arabidopsis* boron transporters through distinct trafficking pathways. Proc Natl Acad Sci U S A 107:5220–5225
Teh OK, Moore I (2007) An ARF-GEF acting at the Golgi and in selective endocytosis in polarized plant cells. Nature 448:493–496
Terasaka K, Blakeslee JJ, Titapiwatanakun B et al (2005) ABCB4, an ATP binding cassette P-glycoprotein, catalyzes auxin transport in *Arabidopsis thaliana* roots. Plant Cell 17:2922–2939
Titapiwatanakun B, Blakeslee JJ, Bandyopadhyay A et al (2009) ABCB19/ABCB19 stabilizes PIN1 in membrane microdomains in *Arabidopsis*. Plant J 57:27–44
Wang B, Bailly A, Zwiewka M et al (2013) Arabidopsis TWISTED DWARF1 functionally interacts with auxin exporter ABCB1 on the root plasma membrane. Plant Cell 25:202–214
Wu G, Lewis DR, Spalding EP (2007) Mutations in *Arabidopsis Multidrug Resistance-Like* ABC transporters separate the roles of acropetal and basipetal auxin transport in lateral root development. Plant Cell 19:1826–1837
Wu G, Otegui MS, Spalding EP (2010) The ER-localized TWD1 immunophilin is necessary for localization of multidrug resistance-like proteins required for polar auxin transport in *Arabidopsis* roots. Plant Cell 22:3295–3304
Zerial M, McBride H (2001) Rab proteins as membrane organizers. Nat Rev Mol Cell Biol 2:107–117

Function of ABCBs in Light Signaling

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Abstract Light signaling controls the growth and development of plants through regulating gene expression and the action of phytohormones including auxin. Among 21 full-size ATP-binding cassette protein subfamily B (*ABCB*) genes in the *Arabidopsis thaliana* genome, *ABCB19*/*PGP19*/*MDR1* (*P-GLYCOPROTEIN19*/*MULTIDRUG RESISTANCE1*) and its closest homolog *ABCB1*/*PGP1* encode auxin transporters, and these functions have been well studied in the light responses of plants. They are involved not only in photomorphogenesis including inhibition of hypocotyl elongation and apical hook opening but also in photomovements including phototropic responses and light-induced randomization of hypocotyl growth orientation. Here, we review the functional regulation of *ABCB19* by light signaling, and then discuss the functions of *ABCB19* and *ABCB1* for each light response.

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1 Light Regulation of *ABCB19* Function

Light signaling controls *ABCB19* expression in *Arabidopsis thalianal* seedlings. Noh et al. (2001) prepared transgenic plants expressing the *β-glucuronidase* (*GUS*) reporter gene driven by the *ABCB19* promoter and observed its expression patterns in Arabidopsis seedlings. GUS staining was observed in the whole hypocotyl—excluding cotyledons—of 5-day-old etiolated seedlings grown in darkness. On the other hand, in de-etiolated seedlings grown under continuous white light (WL) conditions at 80 μmol m^{-2} s^{-1}, GUS staining was observed in the cotyledons, shoot apex, and apical region of hypocotyls, but not in other hypocotyl regions. In situ hybridization using an *ABCB19* antisense probe and immunostaining using anti-ABCB19 antibodies also indicated that *ABCB19* is expressed in the whole hypocotyl of dark-grown seedlings and in cotyledonary nodes and the upper hypocotyl of WL-grown seedlings, and that it is increasingly restricted to vascular tissue in hypocotyl regions distal to the node in WL-grown seedlings (Blakeslee et al. 2007). Nagashima et al. (2008a) examined the effects of red and blue light (RL and BL) exposure on *ABCB19* expression by northern blot and immunoblot analyses. Three-day-old seedlings grown under RL and BL conditions at 50 μmol m^{-2} s^{-1} exhibited remarkable reductions in the transcript and protein levels of *ABCB19* in comparison with dark-grown etiolated seedlings. When 3-day-old etiolated seedlings were irradiated with RL or BL at 50 μmol m^{-2} s^{-1} for 4 h, the amounts of ABCB19 proteins were decreased by half in the upper portions of the hypocotyls. These RL and BL effects depended on the RL photoreceptors phytochrome A (phyA) and phyB and the BL photoreceptors cryptochrome 1 (cry1) and cry2, respectively. These results suggest that such types of light irradiation lead to the suppression of *ABCB19* transcription via phy and cry and reduce its expression in the hypocotyls. As its expression is also auxin responsive (Noh et al. 2001) and light controls the auxin level in Arabidopsis seedlings (Nagashima et al. 2008a; Halliday et al. 2009; Christie et al. 2011), light signaling might also control *ABCB19* expression by regulating auxin levels.

In many cases, types of light irradiation lead to the suppression of *ABCB19* expression in hypocotyls and whole seedlings. However, there are also reports showing that *ABCB19* expression is unaffected by types of light irradiation under some experimental conditions. A northern blot analysis by Lin and Wang (2005) showed that the transcripts were reduced in 4-day-old seedlings grown under far-red light (FR) at 0.5 μmol m^{-2} s^{-1} but not under RL at 30 μmol m^{-2} s^{-1} or BL at 5 μmol m^{-2} s^{-1}, compared with dark-grown etiolated seedlings. Expression analysis of the *ABCB19 promoter::GFP-ABCB19* fusion gene by Wu et al. (2010a) indicated that the green fluorescent protein (GFP) signal was remarkably reduced in the hypocotyl elongation zone of 2-day-old etiolated seedlings by BL irradiation at 50 μmol m^{-2} s^{-1} for 5 h but not by RL irradiation at 100 μmol m^{-2} s^{-1}. Although the exact reason is unclear, growth conditions of seedlings (e.g., age of seedlings and growth medium) and/or the fluence rates of light might influence *ABCB19* expression.

BL signals also modulate the function of the ABCB protein through its phosphorylation. Christie et al. (2011) reported that an activation of the BL photoreceptor phototropin 1 (phot1; see also chapter "Structure–Function of Plant ABC-Transporters"), which harbors a Ser/Thr-type kinase domain and belongs to the AGCVIII protein kinase family with PINOID (PID), WAG, and D6PK (Robert and Offringa 2008; Sakai and Haga 2012), leads to the suppression of basipetal auxin transport in hypocotyls and that phot1 directly phosphorylates ABCB19 protein in vitro and suppresses its auxin transport activity in HeLa cells. Phot1 binds to ABCB19 directly in etiolated seedlings, but BL irradiation disrupts this interaction, suggesting that phot1 inhibits the activity of ABCB19 transiently as an early event of BL effects. Further, coimmunoprecipitation analysis indicated that BL irradiation caused an attenuation of interactions between ABCB19 and an immunophilin-like protein, TWISTED DWARF1 (TWD1), in wild-type seedlings in a phot1-dependent manner (Christie et al. 2011). This interaction is necessary for the auxin export activity of ABCB19 (Geisler et al. 2003; Bailly et al. 2008; Wu et al. 2010b). Thus, phosphorylation of ABCB19 by phot1 likely inhibits its interaction with TWD1 and its auxin transport activity. Another AGCVIII kinase, PID, phosphorylates ABCB1 and modulates its auxin transport activities (Henrichs et al. 2012). Phosphorylation of ABCB19 and ABCB1 by AGCVIII kinases might be among the underlying mechanisms controlling its auxin transport activities (see chapter "It Takes More Than Two to Tango: Regulation of Plant ABC Transporters").

There are also other possibilities for the ways in which types of light can regulate the function of ABCB19. For example, flavonoid accumulation caused by strong light and ultraviolet irradiation can affect the activities of ABCB transporters (Taylor and Grotewold 2005; Petrussa et al. 2013). Flavonoids seem to disrupt the interaction between ABCB transporters and TWD1 and inhibit these auxin transport activities (Bailly et al. 2008). However, the roles of flavonoids in light signal and auxin function have not yet been examined thoroughly. Alterations of ABCB subcellular localizations in response to light have also not been reported. At least, the cellular localization of GFP-ABCB19 fusion proteins is apolar in both hypocotyls of dark-grown etiolated and WL-grown de-etiolated seedlings (Wu et al. 2010a), and types of light irradiation have no effect on the subcellular localization of ABCB19.

2 Functions of ABCB1 and ABCB19 in Photomorphogenesis

Jensen et al. (1998) reported an inhibitory effect of the auxin transport inhibitor 1-naphthylphthalamic acid (NPA) on hypocotyl elongation in de-etiolated seedlings under continuous WL conditions but not in etiolated seedlings under darkness. This finding suggests that auxin transport activity has a more important role in

elongation responses in light-grown seedlings than in dark-grown seedlings of Arabidopsis. Noh et al. (2001) presented direct evidence that ABCB1 and ABCB19 participate in basipetal auxin transport in hypocotyls, suggesting that they are involved in hypocotyl elongation under light exposure conditions. In agreement with this scenario, *abcb1* and *abcb19* mutants show some impairments of hypocotyl elongation under light conditions compared with dark conditions. Lin and Wang (2005) reported that 4-day-old seedlings of *abcb1* and *abcb19* mutants grown under the continuous irradiation of FR, RL, and BL show shorter hypocotyls than in wild-type seedlings but that mutant seedlings grown under darkness show similar patterns of hypocotyl elongations as the wild type. Sidler et al. (1998) reported that expression of the *ABCB1* antisense cDNA and overexpression of the *ABCB1* gene caused either suppression or enhancement of hypocotyl elongation, respectively, in 14- to 19-day-old seedlings grown under continuous WL, FR, RL, and BL. Wu et al. (2010a) reported that a loss-of-function mutation of *ABCB19* and overexpression of the *ABCB19* gene caused suppression or enhancement of hypocotyl elongation, respectively, in 7-day-old seedlings grown under continuous RL. Although (against expectation) the *abcb19* mutants showed almost the same degree of hypocotyl elongation as wild-type seedlings under continuous BL exposure, the *ABCB19*-overexpressing lines showed longer hypocotyls (Wu et al. 2010a). These effects were observed under light irradiations with moderate fluence rates (WL at 20 μmol m^{-2} s^{-1}, RL at 25–50 μmol m^{-2} s^{-1}, BL at 5–15 μmol m^{-2} s^{-1}, and FR at 0.05–05 μmol m^{-2} s^{-1}), but not under weak or strong light irradiation (Sidler et al. 1998; Noh et al. 2001; Lin and Wang 2005). The *abcb1 abcb19* double mutants show a dwarf phenotype and a suppression of hypocotyl elongation in both light-grown and dark-grown seedlings (Noh et al. 2001; Geisler et al. 2003), suggesting that their auxin transport activities play some roles in hypocotyl elongation in both light-grown and dark-grown Arabidopsis seedlings. However, further contributions of ABCB1 and ABCB19 to hypocotyl elongation are probably generated under light irradiations with moderate fluence rates. Thus, the activities of ABCB1 and ABCB19 act in hypocotyl elongation positively, and their contributions to hypocotyl elongation can increase under light conditions in a fluence rate-dependent manner.

How do ABCB1 and ABCB19 function in hypocotyl elongation of seedlings? Auxin levels are closely tied to hypocotyl elongation in Arabidopsis seedlings. Thus, auxin overproduction in Arabidopsis mutants including *yucca* (Zhao et al. 2001), *sur1*/*rooty* (Boerjan et al. 1995; King et al. 1995), *sur2*/*red* (Delarue et al. 1998), and cytochrome P450 CYP79B2-overexpressing lines (Zhao et al. 2002) show long hypocotyl phenotypes, and an auxin biosynthesis mutant *taa1*/*sav3* shows a short hypocotyl phenotype under light conditions (Tao et al. 2008). As the *abcb19* mutant seedlings show low indole-3-acetic acid (IAA) levels (Bailly et al. 2008; Nagashima et al. 2008a; Christie et al. 2011) and the *ABCB19*-overexpressing lines likely show higher IAA levels in hypocotyls/aerial parts of seedlings (Wu et al. 2010a), those alterations might lead to short or long hypocotyls in respective mutant lines. It is not known why the degree of dependence on ABCB1 and ABCB19 is increased in hypocotyl elongation responses by

light irradiation patterns. This anomaly is similar to the question of why auxin transport activity has a more important role in elongation responses in light-grown seedlings than in dark-grown seedlings of Arabidopsis (Jensen et al. 1998). A negative effect of light irradiation on *ABCB19* expression does not appear to be involved in this light response, because it rather seems to reduce the degree of dependence on ABCB19. As irradiation with both RL and BL decreases auxin levels in aerial parts of Arabidopsis seedlings (Nagashima et al. 2008a), the auxin supply from shoot/cotyledon to the hypocotyl elongation zone by ABCB1 and ABCB19 might have more important roles in the elongation responses of hypocotyls in light-grown seedlings.

ABCB1 and ABCB19 also appear to be involved in other forms of photomorphogenesis in addition to hypocotyl elongation. Thus, *abcb19* mutants showed a similar cotyledon size to that of wild-type seedlings before germination, but showed slow postgermination cotyledon expansion under light conditions (Lewis et al. 2009). WL irradiation induces ABCB19 expression in cotyledons and petioles (Noh et al. 2001), and its auxin transport activities in cotyledons and petioles likely contribute to cotyledon expansion (Lewis et al. 2009). ABCB1 and ABCB19 are also involved in the hook opening of photomorphogenesis. Although the *abcb19* single mutants showed normal formation and opening of hooks, the *abcb1 abcb19* hook was not as tightly closed in darkness as wild type, and it opened more slowly in response to BL irradiation (Wu et al. 2010a). Auxin asymmetrically accumulates in the concave side of the hook, enduring its differential growth in etiolated seedlings (auxin leads to the suppression of cell elongation in the concave side of the hook), and light irradiation dissipates this asymmetric distribution as the hook opens (Li et al. 2004; Wu et al. 2010a). ABCB1 and ABCB19 probably function in the establishment and dissipation of the asymmetric auxin distribution in hooks of etiolated Arabidopsis seedlings.

Lin and Wang (2005) reported that the contents of chlorophyll and anthocyanin were slightly decreased in *abcb19* seedlings. However, their data were based on individual seedlings, which have small cotyledons and hypocotyls, as described above. Thus, the *abcb19* mutation might indirectly decrease the contents of chlorophyll and anthocyanin through growth reductions in the seedlings. They also reported that the transcription levels of several light-responsive genes including *RBCS* (encoding the small subunit of ribulose-1,5-bisphosphate carboxylase), *CAB3* (encoding the chlorophyll *a*/*b*-binding protein), and *CHS* (encoding chalcone synthase, required for anthocyanin accumulation) decreased in *abcb1* and *abcb19* seedlings under FR conditions. However, those decreases were also observed in dark-grown seedlings, and it is difficult to establish whether ABCB1 and ABCB19 have direct roles in the transcriptional regulation of such light-responsive genes.

3 Function of ABCB19 in Phototropism

Phototropism allows plants to change their growth direction in response to the location of the light source (for reviews see Sakai and Haga 2012; Christie and Murphy 2013). The Cholodny–Went theory suggests that asymmetric auxin distribution occurs in response to unilateral light irradiation as the result of lateral auxin movement and causes differential growth on the two sides of the plant organ leading to bending. In Arabidopsis seedlings, unilateral BL irradiation causes positive hypocotyl phototropism and negative root phototropism. The BL photoreceptors phot1 and phot2 are essential to induce such phototropic responses (Sakai and Haga 2012; Christie and Murphy 2013), and asymmetric auxin distribution caused by unilateral BL irradiation occurs in a phot signal-dependent manner (Haga et al. 2005). There are two important auxin transporters now known to cause the asymmetric auxin distribution in response to BL irradiation. One is PIN-FORMED3 (PIN3), of which the proteins show an apolar localization in endodermal cells under dark conditions and are greatly decreased in the outer lateral side of the endodermal cells on the BL-irradiated hypocotyl side in a phot1-dependent manner (Ding et al. 2011). Such polarization in the irradiated hypocotyl side appears to redirect auxin back into the vasculature and to block the auxin flow from the vasculature to the outer cell layers, and leads to an asymmetric auxin distribution. Genetic analyses have suggested that PIN1, PIN4, and PIN7 also play some roles in hypocotyl phototropism in addition to PIN3 (Haga and Sakai 2012; Willige et al. 2013), although their specific functions in phototropism are unknown. The other transporter is ABCB19, which modulates phototropic responses.

ABCB1 and ABCB19 are necessary for basipetal auxin transport in hypocotyls (Noh et al. 2001), and inhibition of basipetal auxin transport by NPA leads to the suppression of phototropic responses (Friml et al. 2002; Nagashima et al. 2008b). This suggests that ABCB1 and ABCB19 play important roles in hypocotyl phototropism. However, hypocotyl phototropism even in the *abcb1 abcb19* double and *twd1* mutants, in addition to the *abcb19* mutants, appears normal (Nagashima et al. 2008a). Rather, etiolated *abcb19* seedlings exhibited larger hypocotyl curvatures than wild-type seedlings when treated with unilateral BL irradiation at 0.07 μmol m^{-2} s^{-1} (Noh et al. 2003). Pulse-induced, first-positive phototropism of etiolated hypocotyls, continuous light-induced phototropism of dark-acclimated de-etiolated hypocotyls, and root phototropism were all enhanced by the *abcb19* mutation (Christie et al. 2011; Haga and Sakai 2012; Wan et al. 2012), suggesting that the presence of ABCB19 inhibits phototropic responses in Arabidopsis seedlings in any conditions. Additional mutations of *abcb1 abcb4*, *pin1*, *pin3*, or *pin3 pin7* did not influence its effect of enhancing phototropic responses (Nagashima et al. 2008b; Christie et al. 2011; Haga and Sakai 2012). These results strongly suggest that basipetal auxin transport through ABCB1 and ABCB19 is unnecessary for hypocotyl phototropism; rather, ABCB19 suppresses it independently of other auxin transporters.

Christie et al. (2011) have revealed one of the molecular functions of ABCB19 in hypocotyl phototropism. They observed an alteration of the auxin distribution pattern in hypocotyls of dark-acclimated, de-etiolated seedlings in response to phototropic stimuli. They found that unilateral BL irradiation led to the suppression of basipetal auxin transport in the vasculature of hypocotyls in a phot1-dependent manner and to an increase in auxin accumulation in the epidermal tissues of the shaded side of upper hypocotyls. The phot1 BL photoreceptor kinase physically and transiently interacted with ABCB19 in vivo, directly phosphorylated it in vitro, and inhibited its auxin transport activity in coexpressing HeLa cells (Christie et al. 2011), suggesting that activation of phot1 leads to the suppression of basipetal auxin transport through phosphorylation of ABCB19 and an inhibition of its activity. Phot1 inhibition of ABCB19 or a defect in ABCB19 causes auxin accumulation in the shoot apex, which probably increases the asymmetric auxin distributions caused by the subsequent lateral auxin fluxes.

There are still questions remaining on the roles of ABCB19 and PINs in basipetal auxin transport and in lateral auxin fluxes during phototropic responses. PIN1 appears to function coordinately with ABCB19 in basipetal auxin transport in the hypocotyl (Noh et al. 2003; Blakeslee et al. 2007), and the growth rate of *pin1* hypocotyls is lower than that in wild-type seedlings (Haga and Sakai 2012), like that of *abcb19* (Christie et al. 2011). However, mutations cause the opposite effects in hypocotyl phototropism (Haga and Sakai 2012). Whether the *pin1* mutants show auxin accumulation in the shoot apex requires further study. Moreover, the molecular mechanisms of lateral auxin fluxes in the shoot apex remain unclear. Because the phototropic responses are impaired severely in hypocotyls of the *pin1 pin3 pin7* and *pin3 pin4 pin7* triple mutants (Haga and Sakai 2012; Willige et al. 2013), PIN auxin transporters might function redundantly in lateral auxin fluxes. Interestingly, NPA inhibits the phototropic responses in hypocotyls in an ABCB19-dependent manner (Nagashima et al. 2008b), suggesting that NPA inhibits the asymmetric auxin distribution in hypocotyl phototropism through ABCB19. ABCB19 physically interacts with PIN proteins and modulates these auxin transport activities (Blakeslee et al. 2007; Titapiwatanakun et al. 2009), so it might also suppress lateral auxin fluxes of PINs under the presence of NPA. Furthermore, Wan et al. (2012) reported that *abcb19* mutants also exhibited enhanced phototropic root curvature. Molecular function of ABCB19 in root phototropism should also be examined in future studies.

4 Function of ABCB19 in the Straight Growth of Hypocotyls

Irradiation with RL leads to a randomization of hypocotyl growth orientation, which is dependent on phyA and phyB (Robson and Smith 1996; Hangarter 1997). Irradiation with BL also leads to a similar alteration of hypocotyl growth

in a phyA-, cry1-, and cry2-dependent manner in conjunction with defects in the activities of the phototropins (Lariguet and Fankhauser 2004; Ohgishi et al. 2004). Our group isolated the *flabby* mutant, which exhibits enhanced hypocotyl bending induced by activations of phy and/or cry in Arabidopsis, and revealed that the *FLABBY* gene encodes ABCB19 (Nagashima et al. 2008a). Taken together with the enhancing effects of the *ABCB19* mutation on phototropic and gravitropic hypocotyl curvatures (Noh et al. 2003), these results strongly suggest that ABCB19 contributes to the suppression of differential growth and to maintenance of the straight growth of hypocotyls. Thus, phy and cry appear to induce a randomization of hypocotyl growth orientation by two distinct ways: One is through to be the suppression of hypocotyl gravitropism (Robson and Smith 1996; Hangarter 1997; Lariguet and Fankhauser 2004; Ohgishi et al. 2004). Supporting this model, RL irradiation led to the conversion of starch-filled gravity-sensing endodermal amyloplasts to other plastids with chloroplastic or etioplastic features, which caused the suppression of hypocotyl gravitropism in Arabidopsis (Kim et al. 2011). The other possibility is the release from a straight growth pattern. Phy and cry both suppress the expression of *ABCB19* that contributes to the straight growth of hypocotyls as mentioned above. Gibberellin also seems to contribute to the straight growth of Arabidopsis hypocotyls, and phy and cry suppress its effect by the reduction of gibberellin and by other unknown mechanisms, at least under BL conditions (Tsuchida-Mayama et al. 2010). These functions of phy and cry probably enhance not only a randomization of hypocotyl growth orientation under RL conditions but also phototropic responses of hypocotyls under BL conditions.

The *abcb19* mutants exhibit enhanced hypocotyl bending under RL conditions at a large dynamic range of fluence rates from 0.01 to 10 μmol m^{-2} s^{-1} (Nagashima et al. 2008a). Although the *abcb1* single mutants do not show any abnormal phenotypes, both the *abcb1 abcb19* double mutant and the *twd1* single mutant exhibited a remarkable level of hypocotyl bending compared with the *abcb19* single mutant, and often displayed coil-like hypocotyl structures under RL conditions (Nagashima et al. 2008a). In addition, even in the dark, *abcb1 abcb19* and *twd1* mutants exhibited enhanced wavy or distorted hypocotyls (Nagashima et al. 2008a). These data suggest that ABCB19 and ABCB1 partially share a function in the straight growth of hypocotyls and that TWD1 is involved in both processes.

The coil-like hypocotyl growth of *abcb1 abcb19* and *twd1* mutants under RL conditions clearly suggests the presence of some mechanisms promoting hypocotyl bending, in addition to suppression of the gravitropic response and the straight growth of hypocotyls (Nagashima et al. 2008a). When 2-day-old etiolated wild-type seedlings were irradiated with RL at 10 μmol m^{-2} s^{-1}, their hypocotyls started to bend toward the abaxial side of the hypocotyl hook (Fig. 1). This continued for at least 6 h after the start of RL irradiation, and then the growth direction became unclear because of hook opening and, probably, the suppression of hypocotyl gravitropism (Fig. 1). The *abcb19* mutants exhibited enhancement of directional hypocotyl bending (Fig. 1). Expression analysis of the auxin reporter gene *DR5::GUS* suggested that auxin accumulates asymmetrically not only in the concave

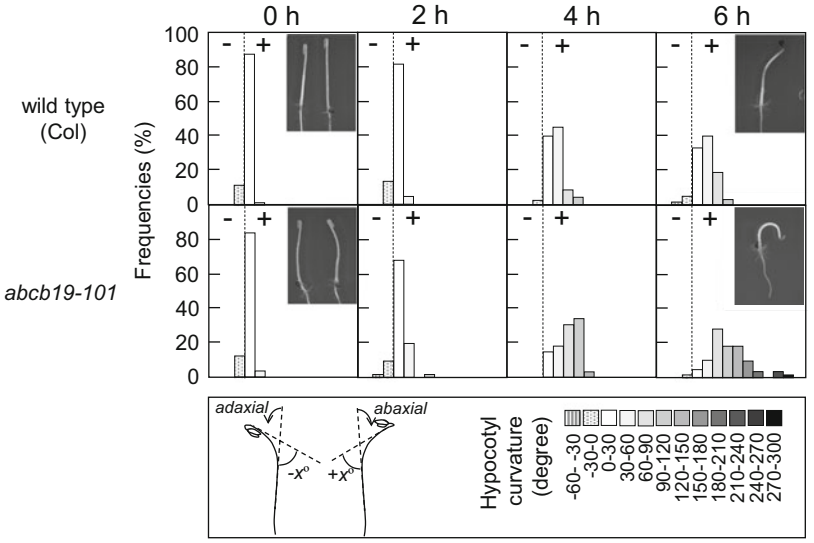

Fig. 1 Red light-induced hypocotyl bending of etiolated wild-type Arabidopsis seedlings and the *abcb19* mutant**.** Two-day-old etiolated seedlings were irradiated with red light at 10 μmol m^{-2} s^{-1}. Frequencies (%) of hypocotyl curvatures at intervals of 30° are indicated by the lengths of the bars (60–81 seedlings were measured in each experiment)

region of the apical hook of etiolated seedlings (Li et al. 2004) but also in the adaxial side of hypocotyls extended toward the elongation zone (Nagashima et al. 2008a, b). Irradiation with RL releases hypocotyls from the gravitropic response and straight growth, and auxin accumulating in the adaxial side of the hook might lead to cell elongation in the adaxial side and promote bending of the hypocotyl toward the abaxial side of the hook in wild-type seedlings. Excess auxin in the adaxial side of the hypocotyl elongation zone may lead to enhanced hypocotyl bending in *abcb19* mutants.

5 Conclusions

Light signaling controls *ABCB19* expression and its auxin transport activity. Interestingly, the expression of the ABCB19 protein is suppressed not only by light stimulation but also by gravistimulation (Nagashima et al. 2008a). Plants can modulate the auxin transport activities of ABCB19 in response to various environmental stimuli and alter their growth patterns. ABCB1 and ABCB19 have dual functions in auxin transport: they are both auxin transporters directly and modulators of PIN auxin transporters. It will be interesting to find out whether ABCB19 modulates activities of other PINs, especially PIN3 and PIN7, for further understanding of the relationship between light and auxin transport.

References

Bailly A, Sovero V, Vincenzetti V et al (2008) Modulation of P-glycoproteins by auxin transport inhibitors is mediated by interaction with immunophilins. J Biol Chem 283:21817–21826

Blakeslee JJ, Bandyopadhyay A, Lee OR et al (2007) Interactions among PIN-FORMED and P-glycoprotein auxin transporters in *Arabidopsis*. Plant Cell 19:131–147

Boerjan W, Cervera M-T, Delarue M et al (1995) *superroot*, a recessive mutation in *Arabidopsis*, confers auxin overproduction. Plant Cell 7:1405–1419

Christie JM, Murphy AS (2013) Shoot phototropism in higher plants: new light through old concepts. Am J Bot 100:35–46

Christie JM, Yang H, Richter GL et al. (2011) phot1 inhibition of ABCB19 primes lateral auxin fluxes in the shoot apex required for phototropism. PLoS Biol 9(6): e1001076. doi: 10.1371/journal.pbio.1001076

Delarue M, Prinsen E, Onckelen V et al (1998) *sur2* mutations of *Arabidopsis thaliana* define a new locus involved in the control of auxin homeostasis. Plant J 14:603–611

Ding Z, Galván-Ampudia CS, Demarsy E et al (2011) Light-mediated polarization of the PIN3 auxin transporter for the phototropic response in Arabidopsis. Nat Cell Biol 13:447–452

Friml J, Wiśniewska J, Benková E et al (2002) Lateral relocation of auxin efflux regulator PIN3 mediates tropism in *Arabidopsis*. Nature 415:806–809

Geisler M, Kolukisaoglu H, Bouchard R et al (2003) TWISTED DWARF1, a unique plasma membrane-anchored immunophilin-like protein, interacts with Arabidopsis multidrug resistance-like transporters AtPGP1 and AtPGP19. Mol Biol Cell 14:4238–4249

Haga K, Sakai T (2012) PIN auxin efflux carriers are necessary for pulse-induced but not continuous light-induced phototropism in Arabidopsis. Plant Physiol 160:763–776

Haga K, Takano M, Neumann R et al (2005) The rice *COLEOPTILE PHOTOTROPISM1* gene encoding an ortholog of Arabidopsis NPH3 is required for phototropism of coleoptiles and lateral translocation of auxin. Plant Cell 17:103–115

Halliday KJ, Martínez-García JF, Josse EM (2009) Integration of light and auxin signaling. Cold Spring Harb Perspect Biol 1:a001586. doi:10.1101/cshperspect.a001586

Hangarter RP (1997) Gravity, light and plant form. Plant Cell Environ 20:796–800

Henrichs S, Wang B, Fukao Y et al (2012) Regulation of ABCB1/PGP1-catalysed auxin transport by linker phosphorylation. EMBO J 31:2965–2980

Jensen PJ, Hangarter RP, Estelle M (1998) Auxin transport is required for hypocotyl elongation in light-grown but not dark-grown Arabidopsis. Plant Physiol 116:455–462

Kim K, Shin J, Lee S-H et al (2011) Phytochromes inhibit hypocotyl negative gravitropism by regulating the development of endodermal amyloplasts through phytochrome-interacting factors. Proc Natl Acad Sci U S A 108:1729–1734

King JJ, Stimart DP, Fisher RH et al (1995) A mutation altering auxin homeostasis and plant morphology in *Arabidopsis*. Plant Cell 7:2023–2037

Lariguet P, Fankhauser C (2004) Hypocotyl growth orientation in blue light is determined by phytochrome A inhibition of gravitropism and phototropin promotion of phototropism. Plant J 40:826–834

Lewis DR, Wu G, Ljung K et al (2009) Auxin transport into cotyledons and cotyledon growth depend similarly on the ABCB19 multidrug resistance-like transporter. Plant J 60:91–101

Li H, Johnson P, Stepanova A et al (2004) Convergence of signaling pathways in the control of differential cell growth in Arabidopsis. Dev Cell 7:193–204

Lin R, Wang H (2005) Two homologous ATP-binding cassette transporter proteins, AtMDR1 and AtPGP1, regulate Arabidopsis photomorphogenesis and root development by mediating polar auxin transport. Plant Physiol 138:949–964

Nagashima A, Suzuki G, Uehara Y et al (2008a) Phytochromes and cryptochromes regulate the differential growth of Arabidopsis hypocotyls in both a PGP19-dependent and a PGP19-independent manner. Plant J 53:516–529

Nagashima A, Uehara Y, Sakai T (2008b) The ABC subfamily B auxin transporter AtABCB19 is involved in the inhibitory effects of N-1-naphthyphthalamic acid on the phototropic and gravitropic responses of Arabidopsis hypocotyls. Plant Cell Physiol 49:1250–1255

Noh B, Murphy AS, Spalding EP (2001) *Multidrug resistance*-like genes of Arabidopsis required for auxin transport and auxin-mediated development. Plant Cell 13:2441–2454

Noh B, Bandyopadhyay A, Peer WA et al (2003) Enhanced gravi- and phototropism in plant *mdr* mutants mislocalizing the auxin efflux protein PIN1. Nature 423:999–1002

Ohgishi M, Saji K, Okada K et al (2004) Functional analysis of each blue-light receptor, cry1, cry2, phot1 and phot2, by using combinatorial multiple mutants in Arabidopsis. Proc Natl Acad Sci U S A 101:2223–2228

Petrussa E, Braidot E, Zancani M et al (2013) Plant flavonoids—biosynthesis, transport and involvement in stress responses. Int J Mol Sci 14:14950–14973

Robert HS, Offringa R (2008) Regulation of auxin transport polarity by AGC kinases. Curr Opin Plant Biol 11:495–502

Robson PR, Smith H (1996) Genetic and transgenic evidence that phytochromes A and B act to modulate the gravitropic orientation of *Arabidopsis thaliana* hypocotyls. Plant Physiol 110:211–216

Sakai T, Haga K (2012) Molecular genetic analysis of phototropism in Arabidopsis. Plant Cell Physiol 53:1517–1534

Sidler M, Hassa P, Hasan S et al (1998) Involvement of an ABC transporter in a developmental pathway regulating hypocotyl cell elongation in the light. Plant Cell 10:1623–1636

Tao Y, Ferrer J-L, Ljung K et al (2008) Rapid synthesis of auxin via a new tryptophan-dependent pathway is required for shade avoidance in plants. Cell 133:164–176

Taylor LP, Grotewold E (2005) Flavonoids as developmental regulators. Curr Opin Plant Biol 8:317–323

Titapiwatanakun B, Blakeslee JJ, Bandyopadhyay A et al (2009) ABCB19/PGP19 stabilises PIN1 in membrane microdomains in Arabidopsis. Plant J 57:27–44

Tsuchida-Mayama T, Sakai T, Hanada A et al (2010) Role of the phytochrome and cryptochrome signaling pathways in hypocotyl phototropism. Plant J 62:653–662

Wan Y, Jasik J, Wang L et al (2012) The signal transducer NPH3 integrates the phototropin1 photosensor with PIN2-based polar auxin transport in *Arabidopsis* root phototropism. Plant Cell 24:551–565

Willige BC, Ahlers S, Zourelidou M et al (2013) D6PK AGCVIII kinases are required for auxin transport and phototropic hypocotyl bending in Arabidopsis. Plant Cell 25:1674–1688

Wu G, Cameron JN, Ljung K et al (2010a) A role for ABCB19-mediated polar auxin transport in seedling photomorphogenesis mediated by cryptochrome 1 and phytochrome B. Plant J 62:179–191

Wu G, Otegui MS, Spalding EP (2010b) The ER-localized TWD1 immunophilin is necessary for localization of multidrug resistance-like proteins required for polar auxin transport in *Arabidopsis* roots. Plant Cell 22:3295–3304

Zhao Y, Christensen SK, Fankhauser C et al (2001) A role for flavin monooxygenase-like enzymes in auxin biosynthesis. Science 291:306–309

Zhao Y, Hull AK, Gupta NR et al (2002) Trp-dependent auxin biosynthesis in *Arabidopsis*: involvement of cytochrome P450s CYP79B2 and CYP79B3. Gene Dev 16:3100–3112

IBA Transport by PDR Proteins

Marta Michniewicz, Samantha K. Powers, and Lucia C. Strader

Abstract Indole-3-butyric acid (IBA) is an important contributor to auxin homeostasis and blocking IBA conversion to the active auxin indole-3-acetic acid (IAA) results in an array of developmental defects. Similar to IAA, IBA movement within the plant is mediated by carriers, many of which remain unidentified. In this chapter, we discuss roles for IBA in plant development, roles for PLEIOTROPIC DRUG RESISTANCE (PDR) members of the ABCG family in IBA transport, and missing transporters required for IBA movement.

1 Coordinated Plant Development Requires Spatial and Temporal Concentrations of Auxin

In plants, endogenous phytohormone-signaling molecules mediate essential communications between cells and tissues. These naturally occurring signals are indispensible for all stages of plant growth and development. Auxin is a prominent plant hormone that regulates cell division and cell elongation to affect plant growth and development (reviewed in Woodward and Bartel 2005). The most abundant and the best-studied naturally occurring active auxin is indole-3-acetic acid (IAA). Decades of study demonstrate that IAA concentration and distribution underpins a broad variety of plant developmental mechanisms and environmental responses ranging from basic cellular processes, such as cell elongation and differential growth, to developmental milestones, such as embryogenesis and organogenesis (reviewed in Perrot-Rechenmann 2010; Sauer et al. 2013). Thus, the establishment of optimum auxin concentration gradients in certain organs, tissues, and cell types is necessary for proper plant development.

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Interestingly, only a small portion of auxin exists as the free, active signaling molecule. IAA homeostasis is tightly regulated by the combination of de novo synthesis (Ljung et al. 2002; Ljung 2013), use of storage forms, and by auxin transport (reviewed in Woodward and Bartel 2005; Normanly 2010; Korasick et al. 2013). The contributions of each of these auxin sources may vary by tissue or in response to various environmental or developmental stimuli. One of the contributors to the pool of free active IAA is naturally occurring auxin precursor, indole-3-butyric acid (IBA).

2 Does IBA Occur Naturally in Plants?

IBA was first described as a synthetic compound affecting auxin-regulated processes; IBA application induces root initiation, influence leaf epinasty, cell division, and stem bending (Zimmerman and Wilcoxon 1935). Nearly 20 years after the discovery of IBA as a "synthetic auxin," Blommaert and colleagues (1954) demonstrated that IBA is an endogenous compound in potato tubers using paper chromatography. Since its initial discovery in potato tubers, IBA has been identified as an endogenous compound in diverse plant species (reviewed in Korasick et al. 2013), including pea epicotyls and cotyledons, cypress leaves, maize leaves, kernels and coleoptiles, tobacco leaves, and Arabidopsis seedlings. The presence of IBA in variety of plants suggest conserved role for this molecule in auxin homeostasis.

Although IBA has been identified as an endogenous compound in many species, the presence of IBA in Arabidopsis, *Populus*, and wheat was recently questioned because it was not detected by GC mass spectrometry in tissues from these plants (Novák et al. 2012). Further complicating identification of IBA in plant tissues, reported IBA concentrations are often at a reduced level compared to IAA concentrations (Sutter and Cohen 1992; Ludwig-Müller et al. 1993, 1997) and IBA levels vary with tissue and growth conditions (Epstein et al. 1989; Ludwig-Müller et al. 1993, 1997). Also, kernels of one maize variety have considerable amount of IBA, whereas IBA was not detectable from kernels of another maize variety (Epstein et al. 1989; Ludwig-Müller et al. 1997), suggesting that IBA also varies by accession. These differences in reported IBA concentrations reflect either true biological differences in IBA accumulation or they may reflect differences in extraction and analysis techniques used to analyze IBA levels. In particular, using Arabidopsis as an example, it is difficult to understand why some methods fail to detect IBA (Novák et al. 2012), others methods detect IBA at low levels (Ludwig-Müller et al. 1993; Strader et al. 2010; Liu et al. 2012b), and IBA-to-IAA conversion mutants display decreased auxin levels (Strader et al. 2011), suggesting that IBA is a major contributor to the auxin pool (see below). Clearly, more research will be necessary to resolve these issues.

3 Many IBA Responses Require IBA-to-IAA Conversion

IBA and IAA are nearly identical compounds; however, IBA has a four-carbon side chain, whereas the active auxin IAA has a two-carbon side chain (Fig. 1). In many plants, the longer side chain of IBA molecule is shortened to produce IAA (reviewed in Strader and Bartel 2011). Peroxisomal IBA-to-IAA conversion is a multistep process similar to fatty acid β-oxidation (Zolman et al. 2000; Strader et al. 2010). Peroxisomal enzymes that appear to be dedicated to IBA β-oxidation include predicted short-chain dehydrogenase/reductase INDOLE-3-BUTYRIC ACID RESPONSE1 (IBR1; Zolman et al. 2008), the acyl-CoA dehydrogenase/oxidase-like IBR3 (Zolman et al. 2007), the predicted enoyl-CoA hydratase IBR10 (Zolman et al. 2008), and ENOYL-COA HYDRATASE2 (ECH2; Strader et al. 2011). Mutants defective in genes encoding these enzymes display resistance to the inhibitory effects of IBA on root elongation and wild-type sensitivity to the active auxin IAA (Zolman et al. 2007, 2008; Strader et al. 2011); consistent with their potential roles in IBA-to-IAA conversion.

IBA-derived auxin contributes to many plant developmental phenotypes. Analysis of higher order IBA conversion mutants revealed decreased free IAA levels, cotyledon expansion defects, root hair expansion defects, reduced apical hook curvature, decreased lateral root production, smaller root meristems, and delayed development in seedlings (Zolman et al. 2008; Strader et al. 2010, 2011). Additionally, Tognetti and colleagues (2010) demonstrated that removing decreasing free IBA levels by overexpressing *UGT74E2* results in increased shoot branching and improved drought and salt tolerance in mature plants. Additionally, treating seedlings with the small molecule naxillin stimulates IBA-to-IAA conversion to result in increased lateral root production (De Rybel et al. 2012). The diverse and severe developmental consequences of altering IBA homeostasis suggest that IBA is a major contributor to the active auxin pool and that IBA-derived auxin impacts many major auxin-related developmental processes.

4 Can IBA Act Also Independently of Its Conversion to IAA?

In many species, IBA is more effective than IAA or synthetic auxins in promoting lateral root and adventitious root formation, causing speculation that IBA itself can act as a signaling molecule in these processes (reviewed in Ludwig-Müller 2000). However, IBA cannot induce lateral roots in Arabidopsis mutants defective in IBA-to-IAA conversion (Zolman et al. 2000, 2007, 2008; Strader et al. 2011), suggesting that the effects of IBA on lateral root induction are through its conversion to IAA. Intriguingly, a recent report suggests that both the IBA-derived auxin and the nitric oxide produced in the peroxisomal IBA-to-IAA conversion process contribute to lateral root induction in Arabidopsis and maize (Schlicht et al. 2013). This nitric

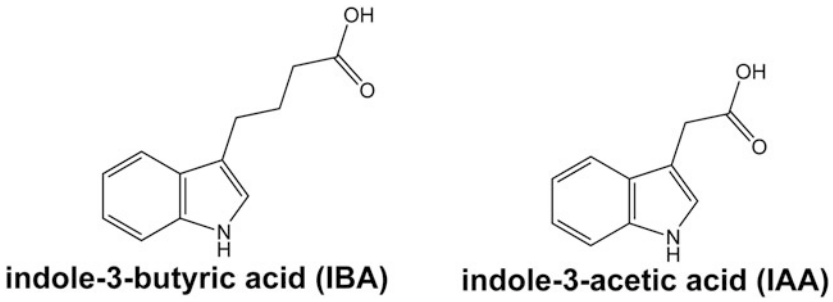

Fig. 1 Structures of the naturally-occurring active auxin IAA (indole-3-acetic acid) and the naturally occurring auxin precursor IBA (indole-3-butyric acid)

oxide produced during IBA-to-IAA conversion may account for the increased effectiveness of IBA compared to other auxins in inducing lateral roots. In addition, the stability of IBA against degradation (Nordström et al. 1991) may contribute to efficiency of IBA promotion of lateral root formation. Although IBA has previously been speculated to be an active lateral root promotion compound, it seems likely that the lateral root initiation observed after IBA application is caused by IBA-derived IAA.

IBA effects on lateral root promotion appear to be through its conversion to active auxin; however, it may be possible that IBA acts as an active signaling molecule under other circumstances. At this point, genetic data in Arabidopsis suggest that the auxin activity of IBA depends fully on its conversion to IAA (reviewed in Strader and Bartel 2011; Spiess and Zolman 2013). In particular, exogenous IBA application to IBA conversion mutants fails to affect seedling growth (Zolman et al. 2007, 2008; Strader et al. 2011), suggesting that, at least in the seedling assays performed, IBA is an inert plant growth regulator unless converted to IAA. Despite evidence that IBA acts through its conversion to IAA, particular stresses induce IBA synthesis and consequently increase the endogenous content of IBA but not IAA (Ludwig-Müller et al. 1995b), suggesting that IBA, but not IAA, plays a roles in response to these stresses. In addition, inoculation of maize roots with an arbuscular mycorrhizal fungus leads to elevated IBA, but not IAA, levels (Ludwig-Müller et al. 1997). Although evidence thus far suggests that IBA plays no developmental role in Arabidopsis outside of its conversion to IAA, it is conceivable that IBA may act as a signaling compound under certain, yet undiscovered circumstances.

5 IBA Transport

Auxin distribution throughout the plant body is achieved by polar cell-to-cell auxin transport in tissues expressing auxin influx and efflux transporters (reviewed in Michniewicz et al. 2007a; Peer et al. 2011). The question arises whether IBA is also

transported throughout plant body and whether IBA transport is similar to IAA transport.

6 Long Distance IBA Transport

Simple bioassay experiments were the first to demonstrate long distance transport of IBA. Early studies demonstrated that application of IBA in one part of the plant affected not only growth of nearby tissues but also auxin-regulated processes distant from the side of IBA application (Zimmerman and Wilcoxon 1935; Went and White 1938; Leopold and Lam 1961; Yang and Davies 1999). These initial observations of IBA effects distant from the application site were later followed with radiolabeled IBA tracking in the plant. Acropetal and basipetal [^{3}H]IBA movement was detected in Cleopatra mandarin midrib sections (Epstein and Sagee 1992) and in Arabidopsis stem cuttings (Ludwig-Müller et al. 1995a). In addition, Rashotte and colleagues (2003) performed a detailed comparison study of radiolabeled IBA and IAA transport in different Arabidopsis tissues, demonstrating that, similar to IAA, roots transport IBA both basipetally (toward the shoot) and acropetally (toward the root tip) and hypocotyls move IBA basipetally (toward the root) but not acropetally (toward the apex). In contrast, inflorescence stems, which transport IAA basipetally, fail to transport IBA (Rashotte et al. 2003). Although these studies provided the first evidence of long distance IBA transport, such interpretation is complicated if we take into consideration IBA-to-IAA metabolism and IBA conjugation to amino acids and sugars (reviewed in Korasick et al. 2013) as well as the fact that radioactive assays alone cannot distinguish between [^{3}H]IBA and its metabolites.

To address concerns about IBA metabolism during transport assays, later studies examined the identity of molecules after the completion of these transport assays (Růžička et al. 2010; Liu et al. 2012a). Using high-performance liquid chromatography (HPLC) analysis, Růžička and colleagues (2010) determined that [^{3}H]IBA application to Arabidopsis root columella cells resulted in identification of [^{3}H]IAA 4 mm above the root tip 2 hours later, consistent with a previous report that Arabidopsis seedlings can convert IBA to IAA after an hour incubation (Strader et al. 2010). Recently, detailed studies utilizing gas chromatography-mass spectrometry (GC-MS) in Arabidopsis hypocotyl and maize coleoptile sections elegantly demonstrated that most of the applied IBA was converted to IAA or ester-linked IBA during transport (Liu et al. 2012a), consistent with a previous report that IBA is metabolized to a polar compound during transport through midrib sections of Cleopatra mandarin (Epstein and Sagee 1992). These studies suggest that the majority of transported IBA is in the form of IBA conjugates or IBA-derived IAA; however, a small amount of free IBA appears to be transported in Arabidopsis cotyledons and maize coleoptiles (Liu et al. 2012a). The directional transport of IBA (or IBA conjugates) in different tissues suggests the existence of active IBA transporters and the differences between IBA and IAA transport suggests that IBA

might use transporters distinct from those of IAA. More detailed studies of IBA transport and IBA transport carriers will be necessary to resolve the exact IBA form carried from cell-to-cell in long distance movement.

7 Many IAA Transporters Do not Transport IBA

IAA enters the cell through the influx carriers and leaves the cell through the efflux carriers to create a net direction flow of auxin through plant tissues (reviewed in Michniewicz et al. 2007a; Peer et al. 2011). This directional IAA distribution allows for local accumulation of auxin to drive developmental processes (reviewed in Vieten et al. 2007). Well-known IAA transporters include amino acid permease-like AUXIN RESISTANT1 (AUX1) mediating IAA influx (Bennett et al. 1996; Yang et al. 2006) and two classes of IAA efflux carriers: the family of PIN-FORMED (PIN) proteins and the MULTIDRUG RESISTANCE/GLYCO-PROTEIN (PGP) class of ATP-Binding Cassette (ABC) transporters (reviewed in Petrášek and Friml 2009). Although IBA is nearly identical to IAA (Fig. 1), IBA and IAA do not appear to share uptake carriers.

IBA uptake in Arabidopsis is a saturable process (Ludwig-Müller et al. 1995a; Rashotte et al. 2003), suggesting that IBA uptake is mediated by carriers rather than passive diffusion. Acropetal and basipetal IBA transport in Arabidopsis roots (Rashotte et al. 2003) and IBA accumulation in excised root tips (Strader and Bartel 2009) are unaltered in *aux1* mutants, suggesting that AUX1 is unlikely to be the IBA uptake carrier. Additionally, IBA does not competitively inhibit [^{3}H] IAA uptake when AUX1 is heterologously expressed in Xenopus oocytes (Yang et al. 2006). However, *aux1* mutants, which are resistant to IAA (Marchant et al. 1999; Yang et al. 2006), are also moderately resistant to IBA (Zolman et al. 2000). This *aux1* IBA resistance may suggest for dual transport activity of AUX1; however, it is likely that the observed IBA resistance in *aux1* likely stems from *aux1* resistance to IBA-derived IAA given the abovementioned evidence that IBA is unlikely to be an AUX1 substrate. Although it is unlikely that AUX1 itself is responsible for IBA uptake, one of the AUX1 family members, consisting of LIKE AUX1 (LAX1), LAX2, AND LAX3 (Swarup et al. 2008), may act as the IBA uptake carrier. For example, IBA displays slight competitive activity against IAA in uptake assays with LAX3 expressed in Xenopus oocytes (Swarup et al. 2008). Future research will be necessary to reveal whether a member of the AUX1 family or some other transporter is responsible for carrier-mediated IBA uptake.

The IBA efflux appears to depend on transporters independent from the PIN and ABCB protein families, which efflux IAA. The polar auxin transport inhibitors naphthylphthalamic acid (NPA) and 2,3,5-triiodobenzoic acid (TIBA), which block IAA efflux and consequently reduce polar transport of IAA (Thomson et al. 1973; Cande and Ray 1976), do not block IBA transport (Rashotte et al. 2003), suggesting that IBA and IAA efflux are through separate mechanisms. In addition, *pin2* mutant roots bend into agar containing the PIN2 substrates IAA and NAA (Utsuno

et al. 1998) because the inability of *pin2* mutants to redistribute IAA and NAA results in a local accumulation on the agar side of the root to cause the root to bend. However, *pin2* mutant roots do not bend toward the agar containing IBA (Poupart and Waddell 2000; Zolman et al. 2000), suggesting that IBA is not a PIN2 substrate and IBA redistribution is functioning in *pin2*. Additionally, basipetal IBA transport is unchanged in *pin2* mutant roots (Rashotte et al. 2003), suggesting that PIN2 is not required for IBA transport. Finally, no examined heterologously expressed IAA transporter, including PIN2, PIN7, ABCB1, and ABCB19, displays IBA efflux activity (Růžička et al. 2010). Collectively, these data suggest that IBA transport is facilitated by carriers independent of those used for IAA transport.

8 Does IBA Has its own Transporters?

IBA undergoes both intercellular (between cells) and intracellular (within the cell) transport into peroxisomes for conversion to the active auxin IAA. Energy-driven active transporters function in both of these IBA transport processes.

9 The ABC Transporter PXA1 May Transport IBA into Peroxisomes

IBA is converted into IAA thorough peroxisomal β-oxidation (reviewed in Strader and Bartel 2011; Spiess and Zolman 2013). Thus, to initiate IBA-to-IAA conversion, IBA needs to be imported into peroxisomes. The carrier that moves IBA into the peroxisome is likely the peroxisomal ABC transporter PEROXISOMAL ABC TRANSPORTER1/ABCD1 (PXA1/ABCD1; see chapter "Plant peroxisomal ABC transporters: flexible and unusual"). Evidence to support PXA1-mediated IBA transport into the peroxisome include: (1) the Arabidopsis PXA1 protein resembles two yeast peroxisomal ABC transporters and four human peroxisomal ABC transporters (Zolman et al. 2001); (2) Arabidopsis *pxa1* loss-of-function mutants are IBA-resistant (Zolman et al. 2001); (3) and Arabidopsis *pxa1* loss-of-function mutants do not efficiently convert IBA to IAA (Strader et al. 2010). At least some *pxa1* mutant phenotypes, including lateral root formation (Zolman et al. 2001) and stamen filament elongation (Footitt et al. 2007), are rescued by exogenous auxin application, suggesting that loss of the PXA1 transporter reduces IBA-to-IAA conversion. Interestingly, in addition to importing IBA into peroxisomes, PXA1 also likely transports fatty acids (reviewed in Linka and Weber 2010) and jasmonic acid precursors into peroxisomes (Theodoulou et al. 2005) for β-oxidation.

After IBA is transported into the peroxisome through the activities of PXA1 and β-oxidized into IAA through the activities of peroxisomal enzymes (see above), IBA-derived IAA would need to be released from the peroxisome by yet unknown

IAA transporter(s). Intriguingly, recent studies demonstrate that PIN family members localize not only to the plasma membrane but also to internal membranes such as the endoplasmic reticulum (Mravec et al. 2009; Ganguly et al. 2010). Thus far, no PIN proteins have been associated with the peroxisome; however, it is possible that peroxisomal IAA effluxer(s) may be members of the PIN or perhaps some other protein family.

10 The PDR Proteins ABCG36 and ABCG37 Serve as IBA Efflux Transporters

Distinct carriers facilitate IBA and IAA transport (Fig. 2). Despite of extensive knowledge about IAA transport and its active transporters, little is known about IBA movement and IBA carriers. The first two transporters identified to facilitate IBA efflux from cells are the ABCG36/PDR8/PEN3 and ABCG37/PDR9/PIS1 proteins which belong to the PLEIOTROPIC DRUG RESISTRANCE (PDR) subclade of the ABCG family of ATP-BINDING CASSETTE transporters (reviewed in Strader and Bartel 2011).

Arabidopsis loss-of-function mutations in *ABCG36* (Strader and Bartel 2009) or *ABCG37* (Strader et al. 2008; Růžička et al. 2010) result in hypersensitivity to exogenous IBA, but not to IAA, in root elongation and lateral root production assays. Because both *abcg36* and *abcg37* are defective in transporters that often efflux substrates, and because both mutants display IBA hypersensitivity, this raises the possibility that the ABCG36 and ABCG37 transporters act to efflux IBA. Supporting the possibility that ABCG36 and ABCG37 efflux IBA, *abcg36* and *abcg37* mutant root tips hyperaccumulate [^{3}H]IBA, but not [^{3}H]IAA, consistent with the IBA and IAA mutant response data (Strader et al. 2008; Strader and Bartel 2009). This [^{3}H]IBA accumulation data suggests that ABCG36 and ABCG37 promote IBA efflux from the root tip. Further, heterologous expression of *ABCG37* directly demonstrate ABCG37 IBA efflux activity; *Schizosaccharomyces pombe* cells expressing *ABCG37* accumulate significant less [^{3}H]IBA than controls, whereas [^{3}H]IAA transport was unchanged compared to control cells (Růžička et al. 2010). In addition, mammalian HeLa cells expressing ABCG37 export [^{3}H]IBA and [^{3}H]2,4-D (Růžička et al. 2010), consistent with IBA and 2,4-D acting as ABCG37 substrates. Although no direct evidence exists that ABCG36 acts as an IBA efflux carrier, the IBA hypersensitivity of *abcg36* mutants (Strader and Bartel 2009) combined with the IBA efflux activity of its homologue ABCG37 (Růžička et al. 2010) support the possibility that IBA is an ABCG36 substrate.

Additionally, ABCG36 and ABG37 seem to work, at least in some aspects, redundantly. Both *abcg36* (Strader and Bartel 2009) and *abcg37* (Strader et al. 2008) mutants are hypersensitive to IBA in root elongation assays and hyperaccumulate [^{3}H]IBA in simplified transport assays, whereas the *abcg36 abcg37* double mutant displays greater IBA hypersensitivity and greater [^{3}H]IBA

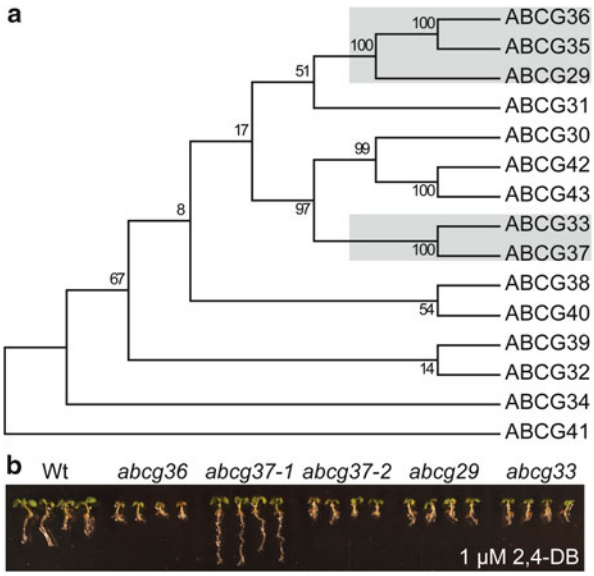

Fig. 2 Mutants in multiple ABCG genes display 2,4-DB hypersensitivity. (**a**) Phylogenetic tree of PDR members of the *Arabidopsis thaliana* ABCG family. Proteins were aligned using ClustalW and the unrooted phylogram was generated using MEGA (Tamura et al. 2011) by performing the bootstrap method with 1,000 replicates. (**b**) Photograph of Col-0 (Wt), *abcg36* (pen3-4; Stein et al. 2006), *abcg37-1* (*pdr9-1*; Ito and Gray 2006), *abcg37-2* (*pdr9-2*; Ito and Gray 2006), *abcg29* (SALK_081047), and *abcg33* (SALK_002380) grown for 8 days on media supplemented with 1 µM 2,4-DB

accumulation than either parent (Růžička et al. 2010). These data suggest that ABCG36 and ABCG37 act redundantly as exporters of IBA from cells. In Arabidopsis, the PLEIOTROPIC DRUG RESISTRANCE (PDR) subclade of the ABCG family of ABC transporters includes 15 members (reviewed in van den Brule and Smart 2002; Crouzet et al. 2006; Verrier et al. 2008). Intriguingly, ABCG36 and ABCG37 are not one another's closest homologue (Fig. 2a) and are only 53 % identical at the amino acid level. Because both *abcg36* and *abcg37* mutants display hypersensitivity to the synthetic auxin precursor 2,4-DB (Strader et al. 2008; Strader and Bartel 2009), we examined insertional alleles in additional *PDR* genes and found that the *abcg29/pdr1* and *abcg33/pdr5* mutants also display hypersensitivity to 2,4-DB (Fig. 2b), suggesting that ABCG29 and ABCG33 in addition to the previously described ABCG36 (Strader and Bartel 2009) and ABCG37 (Ito and Gray 2006; Strader et al. 2008; Růžička et al. 2010), may play roles in transport of auxin-related molecules.

Transport assays and physiological studies indicate that ABCG36 and ABCG37 efflux IBA, but not IAA, from plant cells. However, many members of the PDR transporter family display broad substrates specificity, raising the possibility that ABCG36 and ABCG37 may also serve as a more general efflux carriers.

11 Do ABCG36 and ABCG37 Efflux Exclusively IBA?

Although both ABCG36 and ABCG37 likely efflux IBA but not IAA, it is possible that they also transport additional substrates. Many PDR-type transporters have broad substrate ranges (reviewed in van den Brule and Smart 2002; Crouzet et al. 2006; Verrier et al. 2008) and plant PDR-type ABCG transporters are unlikely to be exceptions. For example, ABCG37 displays broad substrate specificity for various auxinic compounds. *abcg37* mutants display increased sensitivity to many auxinic compounds including synthetic auxins (2,4-D and 2-NOA) and inhibitors of auxin transport such as 1-NOA, NPA, PBA, and TIBA (Fujita and Syōno 1997; Ito and Gray 2006; Růžička et al. 2010). Additionally, *abcg37* mutant root tips hyperaccumulate not only IBA (Strader et al. 2008) but also 2,4-D and NPA (Ito and Gray 2006) in simplified auxin transport assays and *abcg37* protoplasts export significantly less IBA, 2,4-D, and NPA than wild type protoplasts (Růžička et al. 2010), suggesting that those molecules may serve as ABCG37 substrates. Moreover, the heterologous expression of *ABCG37* in HeLa cells conferred significant IBA and 2.4-D export activity (Růžička et al. 2010). Intriguingly, although ABCG36 and ABCG37 may use multiple auxinic compounds as substrates, neither transporter appears to have transport activity on the active auxin IAA, suggesting that their broad substrate specificity is limited.

Notably, ABCG36 and ABCG37 have functions additional to serving as an IBA transporter. Fourcroy and colleagues recently reported that the *ABCG37* gene is upregulated in response to iron deficiency and *abcg37* mutant roots are impaired in coumarin export, suggesting that ABCG37 is required for the coumarin compound secretion (Fourcroy et al. 2014). In addition, *ABCG36* expression is upregulated by cadmium and lead treatment and ABCG36 can serve as an efflux carrier of cadmium and cadmium conjugates in Arabiodpsis (Kim et al. 2007). ABCG36 has been also implicated in pathogen responses. The Arabidopsis *abcg36* loss-of-function mutants exhibited increased sensitivity to some non-host pathogens as well as decreased sensitivity to the Arabidopsis pathogens (Kobae et al. 2006; Stein et al. 2006). Moreover, *abcg36* mutant shoots accumulate less extracellular callose but hyperaccumulate glucosinolate and camalexin in response to pathogens and their elicitors (Bednarek et al. 2009; Clay et al. 2009). Thus, the ABCG36 and ABCG37 transporters appear to have roles in many aspects of plant growth and environmental responses.

12 ABCG36 and ABCG37 Transporters are Polarly Localized Proteins in Root Epidermal Cells

ABCG36 and ABCG37 are enriched at the plasma membrane of the outermost sides of lateral root cap and epidermal cells (Fig. 3; Strader and Bartel 2009; Łangowski et al. 2010; Růžička et al. 2010). Additionally, both proteins also show plasma-

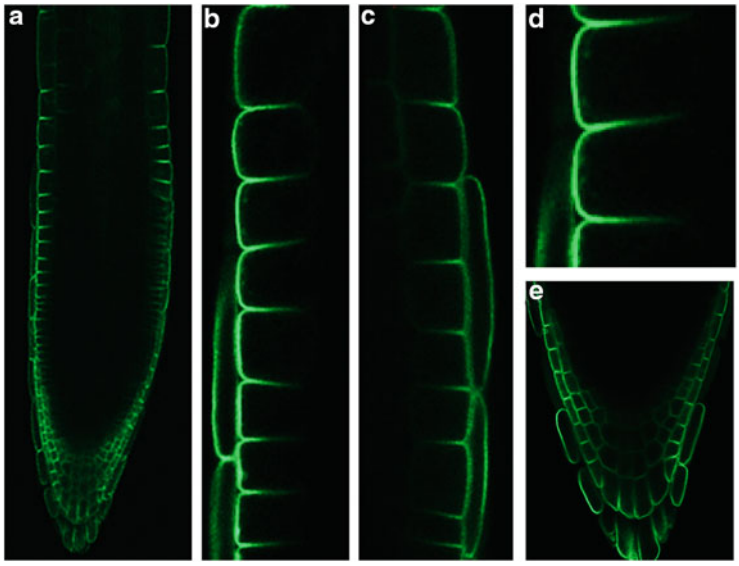

Fig. 3 Confocal images of ABCG36 and ABCG37 localization in 5-day-old Arabidopsis roots. ABCG36-GFP (*pen3-1* carrying *PEN3:PEN3–GFP*; Stein et al. 2006) and GFP-ABCG37 (*pis1-1* carrying *35S:GFP–ABCG37*; Łangowski et al. 2010) both localize to the Outer Polar Domain of root epidermal and lateral root cap cells (Łangowski et al. 2010; Růžička et al. 2010). (**a**, **b**, **d**, **e**) Confocal images of GFP-ABCG37, which accumulates within epidermal and lateral root cap cells (**a**) and magnification of root epidermal cells reveals the polar, outward facing localization of ABCG37 (**b**, **d**). (**e**) Magnification of the lateral root cap shows GFP-ABCG37 localization along the outer edges of the lateral root cap cells regardless of the position and the apical-basal axis of the cells. (**c**) Confocal image of ABCG36-GFP displaying localization to the Outer Lateral Domain of epidermal and lateral root cap cells

membrane localization in root hairs (Łangowski et al. 2010) and ABCG36 is restricted to the plasma membrane of leaf epidermal cells (Kobae et al. 2006; Stein et al. 2006). ABCG36 and ABCG37 localization and activity on IBA efflux are consistent with observed high-auxin root and cotyledon phenotypes of *abcg36* (Strader and Bartel 2009) and *abcg37* (Růžička et al. 2010) mutants, suggesting that ABCG36 and ABCG37 act to reduce auxin levels in the epidermal layer.

The remarkable enrichment of ABCG36 and ABCG37 at the outer face of root epidermal cells suggests that these efflux carriers promote extrusion of IBA into the rhizosphere (Fig. 3). Detailed studies by Łangowski and colleagues (2010), demonstrated that both ABCG36 and ABCG37 transporters are polarly localized to the cell sides facing the root environment regardless of the position and the apical-basal axis of the cells (Fig. 3). Previously defined polar domains of epidermal plant cells include the apical (upper) side enriched in the PIN2 IAA efflux carrier localization and the basal (lower) domain enriched in the ectopically expressed PIN1 IAA efflux carrier (Fig. 4; reviewed in Dettmer and Friml 2011). This polar outward localization of ABCG36 and ABCG37 suggests a novel, distinct, third polar domain within

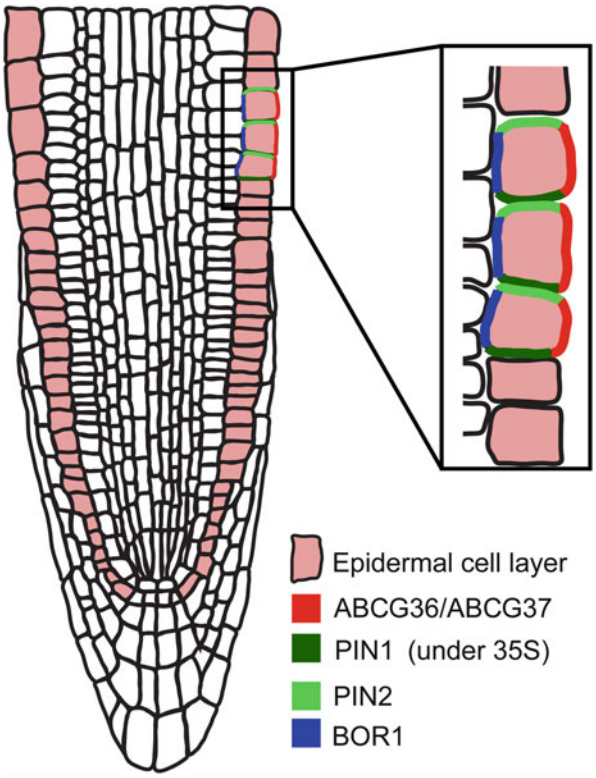

Fig. 4 Cartoon representation of an Arabidopsis root with a magnified epidermal cell layer. The four polar domains of root epidermal cells are marked by different proteins as PIN2 (apical domain; *light green*), ectopically expressed PIN1 (basal domain; *dark green*), BOR1 (inner lateral domain; *blue*), ABCG36 and ABCG37 (outer lateral domain; *red*) all localize in a polar fashion within those cells (reviewed in Strader and Bartel 2009)

epidermal cells that defines the plant interface with its environment. This new polar interface has been termed the "outer lateral domain" (OLD).

Although processes underlying apical and basal polar targeting of protein cargos are well studied, the mechanisms of polar delivery to the lateral side of epidermal cells remain elusive. Intriguingly, many factors required for apical or basal polar localization, such as the ARF GEF GNOM (Geldner et al. 2003; Kleine-Vehn et al. 2006), the actin cytoskeleton (Kleine-Vehn and Friml 2008), BFA-sensitive endocytosis (reviewed in Donaldson and Jackson 2000; Kleine-Vehn and Friml 2008), AXR4 (Hobbie and Estelle 1995; Dharmasiri et al. 2006), the protein kinase PINOID (Friml et al. 2004), and the protein phosphatase PP2A (Michniewicz et al. 2007b), are not required for the maintaining of IBA transporters in the outer polar domain (Łangowski et al. 2010). Because OLD localization appears to be unaffected by altering factors that disrupt apical and basal polar localization

(Łangowski et al. 2010), proteins are likely targeted to the OLD by a distinct yet-to-be-identified mechanism.

13 Why Do ABCG36 and ABCG37 Transporters Localize to the OLD?

The purpose for ABCG36 and ABCG37 extrusion of IBA is not yet understood; however, there are few attractive possibilities for how this IBA efflux could be used by the plant. For example, ABCG36 and ABCG37 may localize to the OLD to limit auxin levels in certain cell types. Consistent with this possibility, *abcg36* and *abcg37* mutants display developmental phenotypes indicative of high auxin levels in certain cell types. For example, both *abcg36* (Strader and Bartel 2009) and *abcg37* (Růžička et al. 2010) loss-of-function mutants display longer root hairs than wild type and *abcg36* mutant seedlings display also enlarged cotyledons (Strader and Bartel 2009). Importantly, these cell expansion phenotypes suggesting elevated auxin levels in *abcg36* are suppressed when *abcg36* is combined with IBA-to-IAA conversion mutants (Strader et al. 2010), suggesting that these cell expansion phenotypes result from increased IBA-derived IAA.

Alternatively, ABCG36 and ABCG37 localization of at the outermost side of root epidermal cells may allow for efflux of compounds, such as IBA or other substrates, into the rhizosphere to participate in plant–microbe or plant–plant interactions. Interestingly, some microorganisms found in soil produce phytohormones to directly enhance plant growth. *Azospirillum spp*. for example, secretes auxin, cytokinins, and gibberellins (reviewed in Bais et al. 2006). This auxin production by bacteria, combined with the IBA extrusion by plants, raises the possibility that root exudates could supply the pool of precursors for microbes to produce active IAA. A more detailed understanding of IBA roles in plant development and microbe interactions will be necessary to begin to understand the effects of IBA efflux by PDR family members on plant growth and environmental responses.

14 Do Other Proteins Display OLD Polar Localization?

The OLD defines the interface between plant and its environment in both above- and belowground tissues. Thus, the OLD exists not only in root but also in aerial portions of the plant as the epidermal domain in leaf and stem tissues that interacts with the environment. This crucial domain likely provides a barrier for selective uptake of nutrients, extrusion of toxic compounds, and exchange of hormonal signals and is the first membrane to encounter abiotic and biotic stresses. Thus

far, only a few proteins have been identified to reside at the OLD of epidermal cells (Fig. 4).

In addition to ABCG36 and ABCG37, the boron efflux transporter BOR4 (Miwa et al. 2007) and the boron uptake carrier NPI5;1 (Takano et al. 2010) also localize to the OLD of root epidermal cells (Fig. 4). Intriguingly, the FK506 binding protein42 TWISTED DWARF1 (TWD1; Bailly et al. 2008) is enriched at the outward-facing side of root epidermal cells (Wang et al. 2013). The ABCG32/PDR4/PERMEABLE CUTICLE1 transporter is also enriched at the outward face of epidermal cells of expanding rosette leaves, top stem segments and petals exports cutin precursors to form the cuticular layer (Bessire et al. 2011). Because several examined PDR members of the ABCG family, including PDR4/ABCG32 (Bessire et al. 2011), PDR8/ABCG36 (Strader and Bartel 2009), and PDR9/ABCG37 (Łangowski et al. 2010; Růžička et al. 2010), are enriched at that OLD, we hypothesize that additional PDR family members will also likely localize to this domain.

15 Are There Additional IBA Transporters?

The active auxin IAA and the auxin precursor IBA appear to use independent transport systems (Fig. 5). Despite broad knowledge about IAA influx and efflux carriers, our understanding of IBA movement is still elusive.

Thus far, only two IBA efflux carriers, ABCG36 (Strader and Bartel 2009) and ABCG37 (Strader et al. 2008; Růžička et al. 2010), have been reported. Both ABCG36 and ABCG37 proteins localize at the plasma membrane of the outermost sides of root epidermal cells and both likely efflux IBA into the rhizosphere (Strader et al. 2008; Strader and Bartel 2009; Łangowski et al. 2010; Růžička et al. 2010). Because these efflux carriers are enriched at the plant–environment interface, they are unlikely to be involved in long-distance IBA movement within the plant. Additionally, *abcg37* mutants do not display defects in long-distance IBA movement (Růžička et al. 2010). Thus, IBA efflux carriers that may mediate transport other than extrusion outside the plant remain unidentified.

Further missing from our understanding of IBA movement are IBA influx carriers. IBA uptake is a saturable process (Ludwig-Müller et al. 1995a; Rashotte et al. 2003), suggesting that IBA uptake is carrier mediated. One possible candidate for the IBA uptake carrier may be the unidentified RIB1 protein because *rib1* mutants display IBA resistance (Poupart and Waddell 2000) and altered IBA (but not IAA) transport (Poupart et al. 2005).

In summary, several aspects of IBA need clarification. Many putative IBA transporters remain unidentified, including the IBA influx carrier and IBA efflux carriers required for long-distance transport. Additionally, the carrier to efflux IBA-derived IAA from the peroxisome is unknown. At the moment, it seems that the bulk of IBA movement through hypocotyls is in the form of conjugates; future research will be needed to determine whether IBA or IBA conjugates move long-distance through roots. It seems certain that identifying these transporters and

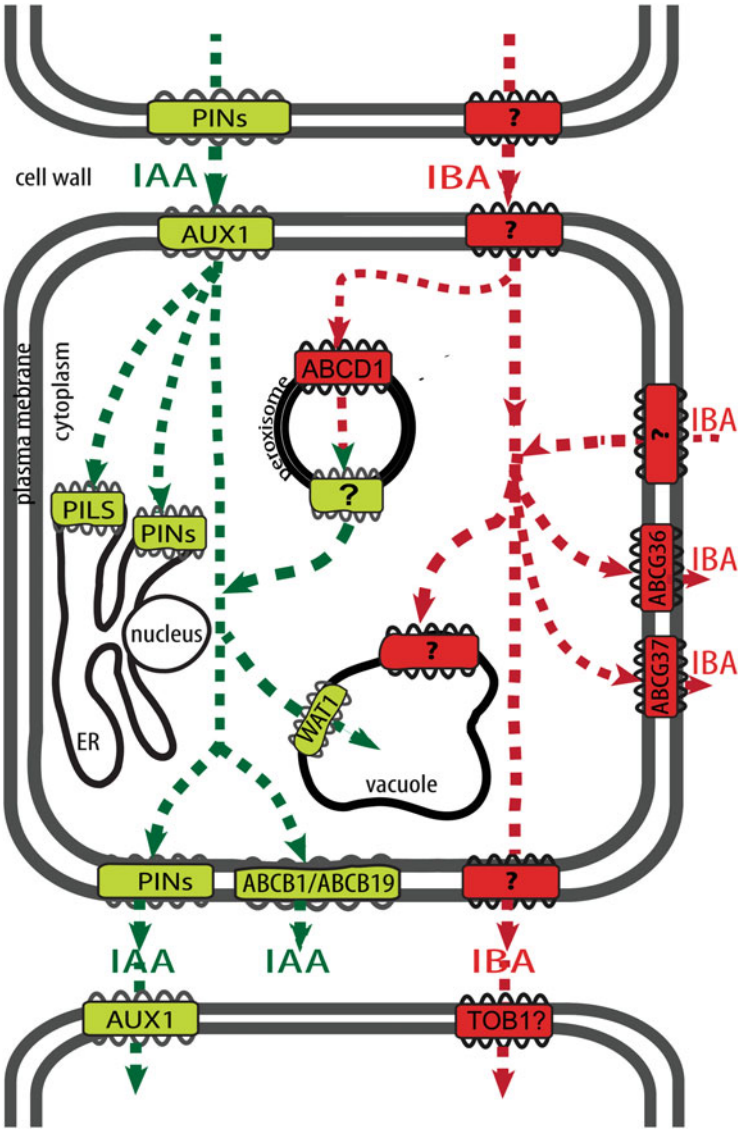

Fig. 5 Cellular model illustrating the complex network of IAA and IBA transporters. The IAA movement and its carriers are marked in *green*, whereas the IBA pathway and its known or predicted transporters are marked in *red*. In addition, protonated IAA can cross the plasma membrane and enter the cell through simple diffusion (not included in the scheme)

clarifying whether IBA or IBA conjugates are moved long distance will illuminate our understanding of roles for IBA-derived auxin and IBA transport in plant development.

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References

Bailly A, Sovero V, Vincenzetti V, Santelia D, Bartnik D, Koenig BW et al (2008) Modulation of P-glycoproteins by auxin transport inhibitors is mediated by interaction with immunophilins. J Biol Chem 283:21817–21826

Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM (2006) The role of root exudates in rhizosphere interactions with plants and other organisms. Annu Rev Plant Biol 57:233–266

Bednarek P, Piślewska-Bednarek M, Svatos A, Schneider B, Doubsky J, Mansurova M et al (2009) A glucosinolate metabolism pathway in living plant cells mediates broad-spectrum antifungal defense. Science 323:101–106

Bennett MJ, Marchant A, Green HG, May ST, Ward SP, Millner PA et al (1996) *Arabidopsis AUX1* gene: a permease-like regulator of root gravitropism. Science 273:948–950

Bessire M, Borel S, Fabre G, Carraça L, Efremova N, Yephremov A et al (2011) A member of the PLEIOTROPIC DRUG RESISTANCE family of ATP binding cassette transporters is required for the formation of a functional cuticle in *Arabidopsis*. Plant Cell 23:1958–1970

Blommaert K (1954) Growth- and inhibiting-substances in relation to the rest period of the potato tuber. Nature 174:970–972

Cande WZ, Ray PM (1976) Nature of cell-to-cell transfer of auxin in polar transport. Planta 129:43–52

Clay NK, Adio AM, Denoux C, Jander G, Ausubel FM (2009) Glucosinolate metabolites required for an *Arabidopsis* innate immune response. Science 323:95–101

Crouzet J, Trombik T, Fraysse AS, Boutry M (2006) Organization and function of the plant pleiotropic drug resistance ABC transporter family. FEBS Lett 580:1123–1130

De Rybel B, Audenaert D, Xuan W, Overvoorde P, Strader LC, Kepinski S et al (2012) A role for the root cap in root branching revealed by the non-auxin probe naxillin. Nat Chem Biol 8:798–805

Dettmer J, Friml J (2011) Cell polarity in plants: when two do the same, it is not the same. Curr Opin Cell Biol 23:686–696

Dharmasiri S, Swarup R, Mockaitis K, Dharmasiri N, Singh SK, Kowalchyk M et al (2006) AXR4 is required for localization of the auxin influx facilitator AUX1. Science 312:1218–1220

Donaldson JG, Jackson CL (2000) Regulators and effectors of the ARF GTPases. Curr Opin Cell Biol 12:475–482

Epstein E, Sagee O (1992) Effect of ethylene treatment on transport and metabolism of indole-3-butyric acid in citrus leaf midribs. Plant Growth Regul 11(4):357–362

Epstein E, Chen K-H, Cohen JD (1989) Identification of indole-3-butyric acid as an endogenous constituent of maize kernels and leaves. Plant Growth Regul 8:215–223

Footitt S, Dietrich D, Fait A, Fernie AR, Holdsworth MJ, Baker A et al (2007) The COMATOSE ATP-binding cassette transporter is required for full fertility in Arabidopsis. Plant Physiol 144:1467–1480

Fourcroy P, Sisó-Terraza P, Sudre D, Savirón M, Reyt G, Gaymard F et al (2014) Involvement of the ABCG37 transporter in secretion of scopoletin and derivatives by Arabidopsis roots in response to iron deficiency. New Phytol 201:155–167

Friml J, Yang X, Michniewicz M, Weijers D, Quint A, Tietz O et al (2004) A PINOID-dependent binary switch in apical-basal PIN polar targeting directs auxin efflux. Science 306:862–865

Fujita H, Syōno K (1997) PIS1, a negative regulator of the action of auxin transport inhibitors in *Arabidopsis thaliana*. Plant J 12:583–595

Ganguly A, Lee SH, Cho M, Lee OR, Yoo H, Cho HT (2010) Differential auxin-transporting activities of PIN-FORMED proteins in Arabidopsis root hair cells. Plant Physiol 153:1046–1061

Geldner N, Anders N, Wolters H, Keicher J, Kornberger W, Muller P et al (2003) The Arabidopsis GNOM ARF-GEF mediates endosomal recycling, auxin transport, and auxin-dependent plant growth. Cell 112:219–230

Hobbie L, Estelle M (1995) The *axr4* auxin-resistant mutants of *Arabidopsis thaliana* define a gene important for root gravitropism and lateral root initiation. Plant J 7:211–220

Ito H, Gray WM (2006) A gain-of-function mutation in the Arabidopsis pleiotropic drug resistance transporter PDR9 confers resistance to auxinic herbicides. Plant Physiol 142:63–74

Kim DY, Bovet L, Maeshima M, Martinoia E, Lee Y (2007) The ABC transporter AtPDR8 is a cadmium extrusion pump conferring heavy metal resistance. Plant J 50:207–218

Kleine-Vehn J, Friml J (2008) Polar targeting and endocytic recycling in auxin-dependent plant development. Annu Rev Cell Dev Biol 24:447–473

Kleine-Vehn J, Dhonukshe P, Swarup R, Bennett M, Friml J (2006) Subcellular trafficking of the Arabidopsis auxin influx carrier AUX1 uses a novel pathway distinct from PIN1. Plant Cell 18:3171–3181

Kobae Y, Sekino T, Yoshioka H, Nakagawa T, Martinoia E, Maeshima M (2006) Loss of AtPDR8, a plasma membrane ABC transporter of *Arabidopsis thaliana*, causes hypersensitive cell death upon pathogen infection. Plant Cell Physiol 47:309–318

Korasick DA, Enders TA, Strader LC (2013) Auxin biosynthesis and storage forms. J Exp Bot 64:2541–2555

Łangowski Ł, Růžička K, Naramoto S, Kleine-Vehn J, Friml J (2010) Trafficking to the outer polar domain defines the root-soil interface. Curr Biol 20:904–908

Leopold AC, Lam SL (1961) Polar transport of three auxins. In: Klein RM (ed) Plant growth regulation. Iowa State University Press, Iowa, pp 411–418

Linka N, Weber AP (2010) Intracellular metabolite transporters in plants. Mol Plant 3:21–53

Liu X, Barkawi L, Gardner G, Cohen JD (2012a) Transport of indole-3-butyric acid and indole-3-acetic acid in Arabidopsis hypocotyls using stable isotope labeling. Plant Physiol 158:1988–2000

Liu X, Hegeman AD, Gardner G, Cohen JD (2012b) Protocol: High-throughput and quantitative assays of auxin and auxin precursors from minute tissue samples. Plant Methods 8:31

Ljung K (2013) Auxin metabolism and homeostasis during plant development. Development 140:943–950

Ljung K, Hull AK, Kowalczyk M, Marchant A, Celenza J, Cohen JD et al (2002) Biosynthesis, conjugation, catabolism and homeostasis of indole-3-acetic acid in *Arabidopsis thaliana*. Plant Mol Biol 50:309–332

Ludwig-Müller J (2000) Indole-3-butyric acid in plant growth and development. Plant Growth Regul 32:219–230

Ludwig-Müller J, Sass S, Sutter EG, Wodner M, Epstein E (1993) Indole-3-butyric acid in *Arabidopsis thaliana* I. Identification and quantification. Plant Growth Regul 13:179–187

Ludwig-Müller J, Raisig A, Hilgenberg W (1995a) Uptake and transport of indole-3-butyric acid in *Arabidopsis thaliana*: comparison with other natural and synthetic auxins. J Plant Physiol 147:351–354

Ludwig-Müller J, Schubert B, Pieper K (1995b) Regulation of IBA synthetase from maize (*Zea mays* L.) by drought stress and ABA. J Exp Bot 46:423–432

Ludwig-Müller J, Kaldorf M, Sutter EG, Epstein E (1997) Indole-3-butyric acid (IBA) is enhanced in young maize (*Zea mays* L.) roots colonized with the arbuscular mycorrhizal fungus *Glomus intraradices*. Plant Sci 125:153–162

Marchant A, Kargul J, May ST, Muller P, Delbarre A, Perrot-Rechenmann C et al (1999) AUX1 regulates root gravitropism in *Arabidopsis* by facilitating auxin uptake within root apical tissues. EMBO J 18:2066–2073

Michniewicz M, Brewer PB, Friml JI (2007a) Polar auxin transport and asymmetric auxin distribution. Arabidopsis Book 5:e0108

Michniewicz M, Zago MK, Abas L, Weijers D, Schweighofer A, Meskiene I et al (2007b) Antagonistic regulation of PIN phosphorylation by PP2A and PINOID directs auxin flux. Cell 130:1044–1056

Miwa K, Takano J, Omori H, Seki M, Shinozaki K, Fujiwara T (2007) Plants tolerant of high boron levels. Science 318:1417

Mravec J, Skůpa P, Bailly A, Hoyerová P, Bielach A (2009) Subcellular homeostasis of phytohormone auxin is mediated by the ER-localized PIN5 transporter. Nature 459:1136–1140

Nordström A-C, Jacobs FA, Eliasson L (1991) Effect of exogenous indole-3-acetic acid and indole-3-butyric acid on internal levels of the respective auxins and their conjugation with aspartic acid during adventitious root formation in pea cuttings. Plant Physiol 96:856–861

Normanly J (2010) Approaching cellular and molecular resolution of auxin biosynthesis and metabolism. Cold Spring Harb Perspect Biol 2:a001594

Novák O, Hényková E, Sairanen I, Kowalczyk M, Pospíšil T, Ljung K (2012) Tissue-specific profiling of the *Arabidopsis thaliana* auxin metabolome. Plant J 72:523–536

Peer WA, Blakeslee JJ, Yang H, Murphy AS (2011) Seven things we think we know about auxin transport. Mol Plant 4:487–504

Perrot-Rechenmann C (2010) Cellular responses to auxin: division versus expansion. Cold Spring Harb Perspect Biol 2:a001446

Petrášek J, Friml J (2009) Auxin transport routes in plant development. Development 136:2675–2688

Poupart J, Waddell CS (2000) The *rib1* mutant is resistant to indole-3-butyric acid, an endogenous auxin in Arabidopsis. Plant Physiol 124:1739–1751

Poupart J, Rashotte AM, Muday GK, Waddell CS (2005) The *rib1* mutant of Arabidopsis has alterations in indole-3-butyric acid transport, hypocotyl elongation, and root architecture. Plant Physiol 139:1460–1471

Rashotte AM, Poupart J, Waddell CS, Muday GK (2003) Transport of the two natural auxins, indole-3-butyric acid and indole-3-acetic acid, in Arabidopsis. Plant Physiol 133:761–772

Růžička K, Strader LC, Bailly A, Yang H, Blakeslee J, Łangowski Ł et al (2010) Arabidopsis *PIS1* encodes the ABCG37 transporter of auxinic compounds including the auxin precursor indole-3-butyric acid. Proc Natl Acad Sci U S A 107:10749–10753

Sauer M, Robert S, Kleine-Vehn J (2013) Auxin: simply complicated. J Exp Bot 64:2565–2577

Schlicht M, Ludwig-Müller J, Burbach C, Volkmann D, Baluska F (2013) Indole-3-butyric acid induces lateral root formation via peroxisome-derived indole-3-acetic acid and nitric oxide. New Phytol 200(2):473–482

Spiess GM, Zolman BK (2013) Peroxisomes as a source of auxin signaling molecules. Subcell Biochem 69:257–281

Stein M, Dittgen J, Sanchez-Rodriguez C, Hou BH, Molina A, Schulze-Lefert P et al (2006) Arabidopsis PEN3/PDR8, an ATP binding cassette transporter, contributes to nonhost resistance to inappropriate pathogens that enter by direct penetration. Plant Cell 18:731–746

Strader LC, Bartel B (2009) The Arabidopsis PLEIOTROPIC DRUG RESISTANCE8/ABCG36 ATP binding cassette transporter modulates sensitivity to the auxin precursor indole-3-butyric acid. Plant Cell 21:1992–2007

Strader LC, Bartel B (2011) Transport and metabolism of the endogenous auxin precursor indole-3-butyric acid. Mol Plant 4:477–486

Strader LC, Monroe-Augustus M, Rogers KC, Lin GL, Bartel B (2008) Arabidopsis *iba response5* (*ibr5*) suppressors separate responses to various hormones. Genetics 180:2019–2031

Strader LC, Hendrickson Culler A, Cohen JD, Bartel B (2010) Conversion of endogenous indole-3-butyric acid to indole-3-acetic acid drives cell expansion in Arabidopsis seedlings. Plant Physiol 153:1577–1586

Strader LC, Wheeler DL, Christensen SE, Berens JC, Cohen JD, Rampey RA et al (2011) Multiple facets of *Arabidopsis* seedling development require indole-3-butyric acid-derived auxin. Plant Cell 23:984–999

Sutter EG, Cohen JD (1992) Measurement of indolebutyric acid in plant tissues by isotope dilution gas chromatography-mass spectrometry analysis. Plant Physiol 99:1719–1722

Swarup K, Benková E, Swarup R, Casimiro I, Péret B, Yang Y et al (2008) The auxin influx carrier LAX3 promotes lateral root emergence. Nat Cell Biol 10:946–954

Takano J, Tanaka M, Toyoda A, Miwa K, Kasai K, Fuji K et al (2010) Polar localization and degradation of Arabidopsis boron transporters through distinct trafficking pathways. Proc Natl Acad Sci U S A 107:5220–5225

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739

Theodoulou FL, Job K, Slocombe SP, Footitt S, Holdsworth M, Baker A et al (2005) Jasmonic acid levels are reduced in *COMATOSE* ATP-binding cassette transporter mutants. Implications for transport of jasmonate precursors into peroxisomes. Plant Physiol 137:835–840

Thomson K-S, Hertel R, Müller S (1973) 1-N-Naphthylphthalamic acid and 2,3,4-triiodobenzoic acid. Planta 109:337–352

Tognetti VB, Van Aken O, Morreel K, Vandenbroucke K, van de Cotte B, De Clercq I et al (2010) Perturbation of indole-3-butyric acid homeostasis by the UDP-glucosyltransferase *UGT74E2* modulates Arabidopsis architecture and water stress tolerance. Plant Cell 22:2660–2679

Utsuno K, Shikanai T, Yamada Y, Hashimoto T (1998) *AGR*, an *agravitropic* locus of *Arabidopsis thaliana*, encodes a novel membrane-protein family member. Plant Cell Physiol 39:1111–1118

van den Brule S, Smart CC (2002) The plant PDR family of ABC transporters. Planta 216:95–106

Verrier PJ, Bird D, Burla B, Dassa E, Forestier C, Geisler M et al (2008) Plant ABC proteins–a unified nomenclature and updated inventory. Trends Plant Sci 13:151–159

Vieten A, Sauer M, Brewer PB, Friml J (2007) Molecular and cellular aspects of auxin-transport-mediated development. Trends Plant Sci 12:160–168

Wang B, Bailly A, Zwiewka M, Henrichs S, Azzarello E, Mancuso S et al (2013) Arabidopsis TWISTED DWARF1 functionally interacts with auxin exporter ABCB1 on the root plasma membrane. Plant Cell 25:202–214

Went F, White R (1938) Experiments of the transport of auxin. Bot Gaz 100:465–484

Woodward AW, Bartel B (2005) Auxin: regulation, action, and interaction. Ann Bot 95:707–735

Yang T, Davies PJ (1999) Promotion of stem elongation by indole-3-butyric acid in intact plants of *Pisum sativum* L. Plant Growth Regul 27:157–160

Yang Y, Hammes UZ, Taylor CG, Schachtman DP, Nielsen E (2006) High-affinity auxin transport by the AUX1 influx carrier protein. Curr Biol 16:1123–1127

Zimmerman PW, Wilcoxon F (1935) Several chemical growth substances which cause initiation of roots and other responses in plants. Contrib Boyce Tompson Inst 7:209–229

Zolman BK, Yoder A, Bartel B (2000) Genetic analysis of indole-3-butyric acid responses in *Arabidopsis thaliana* reveals four mutant classes. Genetics 156:1323–1337

Zolman BK, Silva ID, Bartel B (2001) The Arabidopsis *pxa1* mutant is defective in an ATP-binding cassette transporter-like protein required for peroxisomal fatty acid β-oxidation. Plant Physiol 127:1266–1278

Zolman BK, Nyberg M, Bartel B (2007) IBR3, a novel peroxisomal acyl-CoA dehydrogenase-like protein required for indole-3-butyric acid response. Plant Mol Biol 64:59–72

Zolman BK, Martinez N, Millius A, Adham AR, Bartel B (2008) Identification and characterization of *Arabidopsis* indole-3-butyric acid response mutants defective in novel peroxisomal enzymes. Genetics 180:237–251