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Andreas Vilcinskas *Editor*

Yellow Biotechnology II

Insect Biotechnology in Plant Protection and Industry

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Andreas Vilcinskas
Editor

Yellow Biotechnology II

Insect Biotechnology in Plant Protection and Industry

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Preface

"Yellow Biotechnology" has been introduced an alternative term for insect biotechnology, which is an emerging field in applied entomology. As a complement to Part I, which focuses on the use of insects in drug discovery and preclinical research, Part II considers the applications of insect biotechnology in industrial and food biotechnology, and in modern approaches that allow the sustainable protection of plants.

Industrial (white) biotechnology concerns the biotransformation of raw materials into potentially useful industrial products, predominantly by using microbes and/or enzymes. The industrial and food biotechnology toolbox is now expanding to include novel insect-derived enzymes, such as chitinases and cellulases. The first three chapters of this volume, therefore, cover the use of insect enzymes in food biotechnology and for the conversion of biomass. The deployment of insect enzymes in these fields requires platforms for the large-scale production of recombinant insect proteins. "Optimization of Insect Cell-Based Protein Production Processes:Online Monitoring, Expression Systems, Scale Up", therefore, discusses recent developments in the optimization of heterologous production systems based on insect cells.

Insect biotechnology has been defined as the use of biotechnology to develop insects (or their molecules, cells, organs or associated microorganisms) into products and services for specific applications in medicine, plant protection and industry. "Insect Antenna-Based Biosensors for In Situ Detection of Volatiles" provides an intriguing example of applications involving isolated insect organs, namely the use of individual antennae to develop novel biosensors for the in situ detection of volatiles. Such biosensors can now be used to optimize the application of pheromones in plant protection strategies, and are also being developed for the sensitive detection of drugs and explosives at airports.

Insects that feed on crops or stored products are still the most important competitors for human nutrition, and insects that transmit infectious diseases such as malaria threaten human health on a global scale. Insect biotechnology therefore seeks to explore novel and sustainable strategies to control pest and vector insects, including the development of transgenic plants expressing defense proteins or metabolites, RNAi-based approaches and the sterile male technique. The last four chapters of Part II provide insight into the most recent developments in these areas.

The authors of this volume are members or associates of the first German research program on Insect Biotechnology. The research is funded by the Hessen State Ministry of Higher Education, Research and the Arts via the excellence program LOEWE, is hosted by the Justus-Liebig University of Giessen, and is coordinated by the editor of this book. The generous funding under this program allowed a new department to be established within the Fraunhofer Institute for Molecular Biology and Applied Ecology, focusing on the application of insect biotechnology in projects that are jointly supported by industrial partners such as Dow AgroSciences. The latter contributed to this volume with a chapter discussing transgenic approaches for the control of the western corn rootworm. The growing academic and industrial interest in the biotechnology of insects motivated the editor of this volume to assemble the first treatise on Yellow Biotechnology, which was published in the Springer book series *Advances in Biochemical Engineering and Biotechnology*. Part II now takes the reader further by investigating selected frontiers of insect biotechnology in the fields of industry and plant protection.

Autumn 2013 Andreas Vilcinskas

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Insect-Derived Enzymes: A Treasure for Industrial Biotechnology and Food Biotechnology

Nicole Mika, Holger Zorn and Martin Rühl

Abstract Insects are the most diverse group of organisms on earth, colonizing almost every ecological niche of the planet. To survive in various and sometimes extreme habitats, insects have established diverse biological and chemical systems. Core components of these systems are enzymes that enable the insects to feed on diverse nutrient sources. The enzymes are produced by either the insects themselves (homologous) or by symbiotic organisms located in the insects' bodies or in their nests (heterologous). The use of these insect-associated enzymes for applications in the fields of food biotechnology and industrial (white) biotechnology is gaining more and more interest. Prominent examples of insect-derived enzymes include peptidases, amylases, lipases, and β-D-glucosidases. Highly potent peptidases for the degradation of gluten, a storage protein that can cause intestinal disorders, may be received from grain pests. Several insects, such as bark and ambrosia beetles and termites, are able to feed on wood. In the field of white biotechnology, their cellulolytic enzyme systems of mainly endo-1,4-β-D-glucanases and β-D-glucosidases can be employed for saccharification of the most prominent polymer on earth—cellulose.Keyword · Gluten · beetles · grain pests · cellulose.

Keywords Beetles · Gluten · Grain pests · Hydrolases

Contents

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1 Enzymes in Food Biotechnology

Enzymes are indispensable tools for the production of several traditional food products as well as for the synthesis of food additives, vitamins, and flavor compounds. Without the action of enzymes, the production of cheese, bread, wine, or beer would be unthinkable. Enzymes play a crucial role in the conservation of food, the elimination of possible toxic compounds or antinutritive factors, and the improvement of the texture and processing qualities.

Due to the technical improvements in food production, the increasing importance of convenience food, the increasing number of people suffering from food intolerances, and the steady rise of food allergies, the search for enzymes with novel biochemical properties or improved process characteristics is a highly dynamic field of research.

An enormous demand for industrial enzymes arises from the increasing need for an efficient use of natural resources. According to a recent study of the food and agriculture organisation (FAO) of the United Nations, only about two thirds of the food produced worldwide is consumed by humans. One third, about 1.3 billion tons per annum, is lost during agricultural production, on the way to the consumer, or when discarded by the consumers. Per capita, these postharvest losses amount to approximately 300 kg annually [1]. Novel and improved enzymes will help to overcome this unsatisfying situation.

Until now, the enzymes employed in food production have either been derived from microorganisms such as fungi and bacteria or are of plant or mammal origin. Novel enzymes, such as those from insects or insect-associated microorganisms, are highly demanded by the food industry to reduce food incompatibilities such as celiac disease or to eliminate potential antinutritive factors. A variety of insects offer a broad range of yet unknown opportunities to improve processes in food manufacturing.

2 Insects as a Source for Novel Enzymes

Insects are the most diverse taxonomic animal class on earth, colonizing almost every ecological niche of the planet. To survive in various and sometimes extreme habitats, insects have established diverse biological and chemical systems, such as the production of defense molecules [2, 3], stabilizing proteins [4], or lytic enzymes [5]. In some cases, they also harbor symbiotic microorganisms, which are used as digestive helpers, fodder, or both [[6, 7], see below]. This is possible due to the special metabolic systems or exogenous enzymes of the associated organisms.

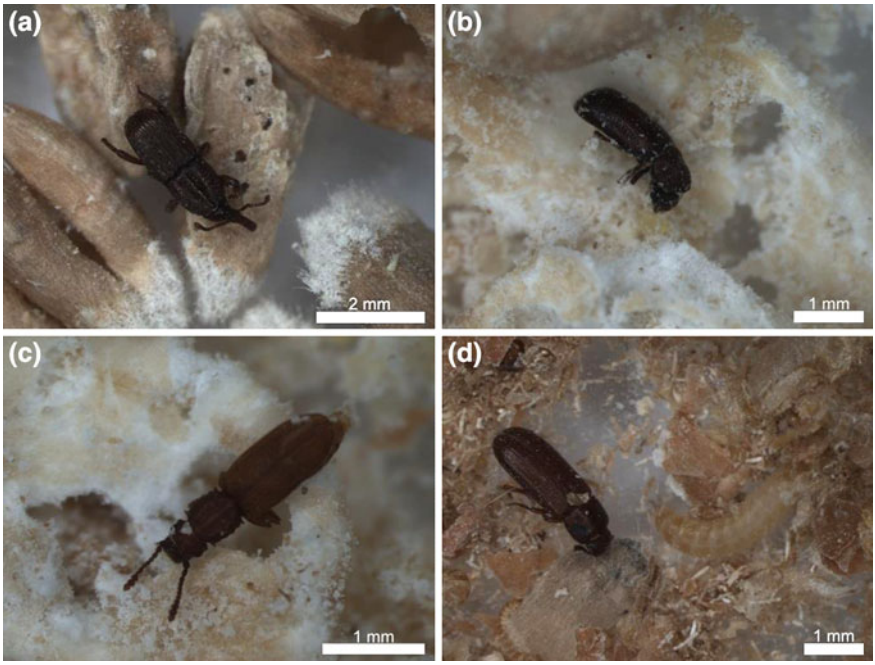


Fig. 1 Examples of grain pests: *S. granaries* (**a**), *R. dominica* (**b**), *O. surinamensis* (**c**), and larvae and adult beetle of *T. castaneum* (**d**)

2.1 Grain Pests

Coleoptera (beetles) represent the largest biodiversity of all creatures [8]. Grain pests in particular are globally distributed. With a short life cycle of only 3–4 weeks, many grain pests are easy to maintain in the laboratory. Because the grain pests rely on the same food sources as humans, their strategies to digest seed proteins may be copied, such as for the production of gluten-free food.

The group of grain pests includes the wheat weevil *Sitophilus granarius*, the lesser grain borer *Rhizopertha dominica*, the sawtoothed grain beetle *Oryzaephilus surinamensis*, the lesser mealworm *Alphitobius diaperinus,* and the red flour beetle *Tribolium castaneum* (Fig. 1).

Grain pests produce various hydrolases, including gylcosidases and peptidases, for the degradation of carbohydrates and storage proteins, respectively, to meet their demand for carbon and nitrogen. α-Amylase activity has been detected in many different grain pests, such as in the moth *Helicoverpa armigera* [9], the sunn pest *Eurygaster integriceps* [10], and in the well-studied flour beetle *T. castaneum* [11]. In addition, various exo- and endo-peptidases have been discovered in numerous insects feeding on cereals: trypsin and chymotrypsin type peptidases in *Plodia interpunctella* [12], a cysteine peptidase in *Tenebrio molitor* [13], and

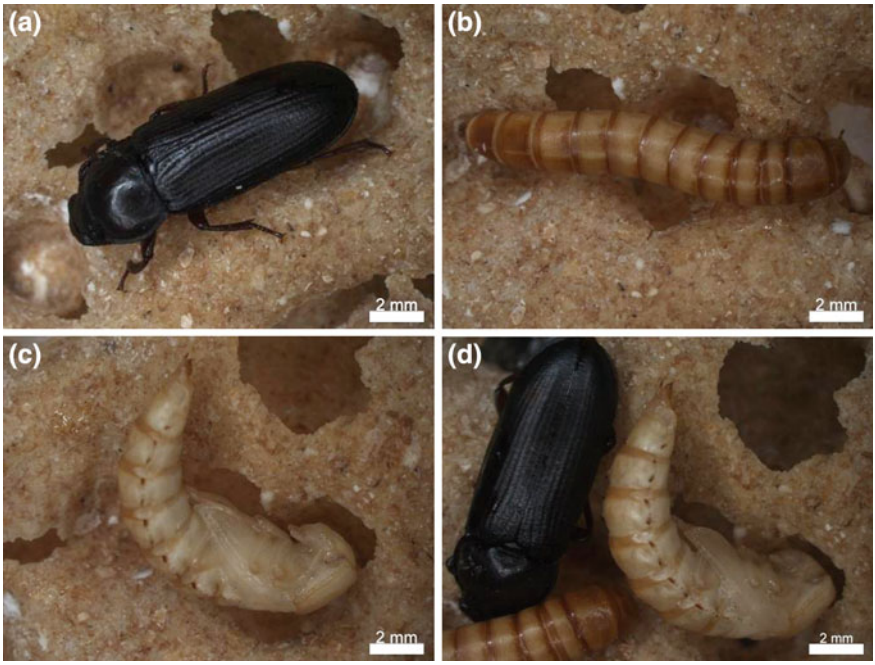


Fig. 2 Adult beetle (**a**, **d**), larva (**b**, **d**) and nymph (**c**, **d**) of *T. molitor*

serine peptidases in *Prostephanus truncates* [14]. One of the best characterized grain pests is the yellow mealworm, *T. molitor* (Fig. 2).

2.2 Gluten-Degrading Enzymes

Celiac disease is an intestinal disorder caused by an uncontrolled immune response on wheat gluten and similar proteins, such as oat, rye, and barley. Typical symptoms of celiac disease include diarrhea, malnutrition, and growth disturbances. Because about 1 % of the worldwide population is suffering from celiac disease [15] and only 100 mg of gluten may cause massive problems to affected individuals [16], gluten-degrading enzymes play an important role in the food industry.

Often, celiac disease is diagnosed in early infancy as a result of the first intake of cereal products. Therefore, almost every baby food manufacturer offers a gluten-free assortment. Adults with celiac disease have to eliminate products such as bread, noodles, cake, and beer from their diets and have to stick to a strict nutrition plan. Although adults can adjust their food to gluten-free products themselves, the range of available products for babies is rather limited. Hence, the production of gluten-free baby food plays an important role in the food industry.

Table 1 Insects' ability to hydrolyze food proteins in zymograms

Grain pest	Casein	Gluten	Rice Protein
Alphitobius diapernius	+	+	n.d.
Oryzaephilus surinamensis	+	+	+
Rhizopertha dominica	+	+	n.d.
Sitophilus granaries	n.d.	n.d.	+
Tenebrio molitor	+	+	+
Tribolium castaneum	–	–	+

+ positive, – negative, n.d. not determined

Possibilities to degrade peptides relevant to celiac disease have been shown in a number of studies. The hydrolysis of celiac disease-associated proteins with enzymes from the ascomycete *Aspergillus niger* [17] and from germinated cereals [18] have been discussed. Although a partial hydrolysis of the target peptides was achieved, more effective and specific peptidases are needed [18]. An obvious conclusion is to focus on insects, such as grain pests, whose source of food are storage proteins of cereal grains. The major compounds of these storage proteins are prolamins, which cover about 50 % of the total seed protein. They contain 30–50 % glutamine and 10–30 % proline residues [19].

In recent studies, enzyme extracts of the grain pests *A. diapernius*, *S. granaries*, *T. castaneum*, *T. molitor*, *O. surinamensis* and *R. dominica* were tested for their capability to hydrolyze casein, gluten, bovine serum albumin, and rice protein.

Except for *T. castaneum*, all of the tested grain pests were able to hydrolyze the substrates in zymograms (Table 1). The highest peptidase activity was determined for *O. surinamensis*, followed by *R. dominica* and *T. molitor*.

To localize the peptidase activities, the insects' ability to hydrolyze the food proteins was compared between deveined and complete beetles. The peptidase activities of the complete beetles were significantly higher compared to those of the deveined beetles as determined by the azocasein assay. On the other hand, the deveined insect samples showed obvious clearing zones in the zymograms as well (Fig. 3). Possibly, the deveined samples still contained traces of gut. Therefore, we conclude that the majority of gluten-degrading enzymes are localized in the insects' gastrointestinal system.

T. molitor is one of the best-examined grain pests. Therefore, the digestive peptidases of *T. molitor* were among the first insect peptidases studied [20, 21].

The digestion of storage proteins in larvae of *T. molitor* occurs in the midgut. Studies by Vinokurov et al. [13] and Elpedina and Goptar [21] revealed a sharp pH gradient from 5.6 in the anterior midgut (AM) to 7.9 in the posterior midgut (PM), and the digestive enzymes are arranged to their pH optima in the AM or PM. According to their pH optima, cysteine peptidases and glycosidases are mostly found in the AM, whereas serine peptidases are preferably found in the PM.

In studies by Goptar et al. [22], three post-glutamine cleaving cysteine peptidases were isolated from the yellow mealworm's midgut. The predominance of cysteine peptidases over serine peptidases, especially in coleopteran larvae, is probably due to an adaptation of the insects to cereals that are rich in naturally

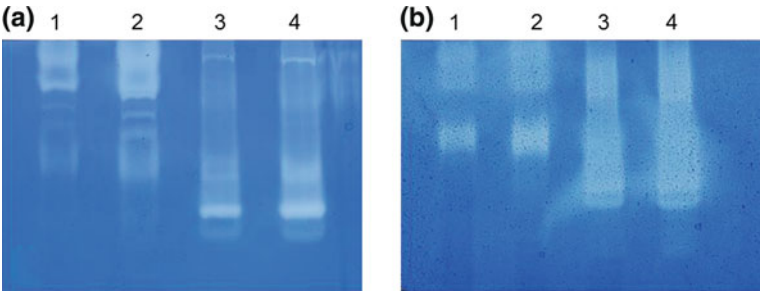


Fig. 3 Comparison of enzyme extracts obtained from deveined (1, 3) and complete beetles (2, 4) of *O. surinamensis* (1, 2) and of *R. dominica* (3, 4). Zymograms contain rice protein (**a**) and gluten (**b**)

occurring inhibitors of serine peptidases [23]. Another explanation goes back to the need of the larvae to digest seed storage proteins rich in glutamine. Hence, specified enzymes to cleave peptide bonds at the glutamine carboxyl group are necessary [22].

A similar arrangement of digestive enzymes was found in the *Tenebroid*-related insects *T. castaneum* and *Tribolium confusum*. A pH range from 5.6–6.0 in the AM to 7.0–7.5 in the PM was determined in the larvae [24]. Approximately 80 % of the total peptidase activity was localized in the AM, whereas the residual 20 % was localized in the PM. The dominating peptidases in *T. castaneum* and *T. confusum* showed approximately 97 % homology to the cysteine peptidases of *T. molitor*.

Konarev et al. [25] purified and characterized a glutenin-specific serine peptidase (GHP) with a molecular weight of about 28 kDa, which belongs to the peptidase-family S1. GHP is specific for the peptide bond between the glutamine and glycine residues in adjacent hexapeptide and nonapeptide motifs, whereas the glutamine-glycine bond was not cleaved within the hexapeptide. This specific property could be used in food processing or for an enzyme therapy.

In summary, several studies focused on insect-derived enzymes for gluten degradation, and various enzymes have been characterized. Insect-derived enzymes with the ability to degrade gluten have a high potential to find future applications in the food industry. Nevertheless, none of these enzymes has made its way to commercialization so far. For that reason, further studies that focus on novel insect-derived enzymes are of special interest.

2.3 Further Insect-Derived Enzymes for Food Biotechnology

2.3.1 Amylases

Amylases, especially α-amylases (EC. 3.2.1.1), belong to the most important digestive enzymes. They occur *inter alia* in human saliva and in the small intestine. Breaking down oligosaccharides of 6–7 glucose units from starch, they may

be compared to endopeptidases. After further incubation, these oligosaccharides can be further hydrolyzed to the disaccharides maltose or isomaltose, or in a final step to glucose.

Apart from α-amylases, β-, gluco- and iso-amylases (EC 3.2.1.2, 3.2.1.3 and 3.2.1.68) are involved in the degradation of starch and glycogen. By cutting maltose from the nonreducing end of a chain, β-amylases are acting as exo-glucosidases. With the ability to hydrolyze α-1 → 6-bonds, isoamylases debranch starch and glycogen.

Currently, amylases are typically obtained from mold fungi or bacteria. They are employed for such purposes as the degradation of starch in wort, the manufacturing of bakery products, and the production of glucose and further sugar specialties.

In the literature, many studies about amylases isolated from insects or insect-associated species can be found. Mehrabadi et al. [26], for example, determined and characterized the α-amylase activity of various grain pests, such as *S. granarius* and *R. dominica*. They showed that the α-amylase activity has been adapted to the acid physiological environment present in larval midguts [27]. Further amylases were described by Saadati Bezdi et al. [28]. They characterized the enzymatic system of salivary glands of the sunn pest *E. integriceps* and identified two α-amylase.

2.3.2 Lipases

Lipases likewise represent an important class of insect-derived enzymes. In the food industry, lipases are used for such purposes as the formation of flavors during the production of cheese and the partial hydrolysis of milk fat during the manufacturing of milk chocolate. Another application is the industrial trans-esterification of oil to yield tailor-made triglycerides. Different from the catalytic hydrogenation, the formation of (*E*)-configured fatty acids is avoided in the enzymatic process. By now, lipases are obtained preferably from microorganisms such as the yeast *Candida lipolytica*. However, a number of studies have already revealed the potential of lipases from insects. Grillo et al. [29] investigated the role of a midgut triacylglycerol-lipase in the lipid metabolism of the hemiptera *Rhodnius prolixus*. The release of free fatty acids catalyzed by this lipase was most efficient between pH 7.0–7.5, and the lipase activity was increased in the presence of salts such as NaCl or $CaCl_2$. Maximum lipase activity was obtained at a NaCl concentration of 0.75 M and a Ca^{2+} concentration of 1.0 M. This characteristic could be useful for special applications in the food industry for which high salt concentrations and/or neutral pH values are needed.

One more enzyme category is widely used in the food industry. Shen et al. [30] isolated and sequenced a pectin methylesterase from the gut of the rice weevil, *Sitophilus oryzae*. In the food industry, pectinolytic enzymes are used for such purposes as the purification and the increase of yields of fruit and vegetable juices.

Pectinases are mostly obtained from fermentation with fungi, but insect-derived pectinolytic enzymes could become an interesting alternative for the food industry.

3 Insect Enzymes for Industrial Biotechnology

Industrial biotechnology, also known as white biotechnology, uses either enzymes or microorganisms to improve already-existing industrial processes or to develop new products and processes demanded by the market. Most of these processes are bulk applications for which large amounts of educts are needed [31]. The availability of the fossil materials gas and oil, which are currently used as educts, is limited. Thus, renewable resources have to be made accessible.

The most important renewable resources are plant polymers, particularly lignocelluloses. Lignocellulose represents the main compound of woody plants, with an estimated annual production of around 200 billion tons [32]. The release of fermentable sugars from lignocelluloses (e.g. wood and straw) for the production of diverse chemicals such as ethanol, butanol, or organic acids is one of the main research areas in white biotechnology. The scale-up of successful pilot experiments to industrial applications is performed in so-called biorefineries (e.g. sunliquid; http://www.bmbf.de/de/17786.php). One of the most important steps towards valuable products from lignocellulose is the initial attack of the complex and cross-linked polymer. For this purpose, oxidative and hydrolytic enzymes are needed.

3.1 Enzymes for the Degradation of Plant Polymers

The most abundant biopolymer on earth is cellulose. It is located in the cell walls of herbaceous plants and in wooden plants together with hemicellulose and lignin. In nature, lignocellulose is mainly degraded by filamentous fungi. They have an efficient oxidative enzymatic system capable of degradation of the complete lignocellulosic polymer into consumable nitrogen and carbon sources [33, 34]. To date, most of the enzymes used for industrial biotechnology are of fungal or bacterial origin. Peroxidases, such as lignin peroxidases (EC 1.11.1.14), Mn-dependent peroxidases (EC 1.11.1.13), versatile peroxidases (EC 1.11.1.13), dye-decolourizing peroxidases (EC 1.11.1.x), and laccases (EC 1.10.3.2), together with supporting enzymes, are able to oxidize and degrade lignin, whereas hydrolytic enzymes are secreted to break down cellulose and hemicelluloses. Cellulases including endo-1,4-β-D-glucanase (EC 3.2.1.4) and exo-1,4-β-D-glucanases (EC 3.2.1.91, EC 3.2.1.176) convert cellulose into di- and oligosaccharides, which in turn are hydrolyzed by β-D-glucosidases (EC 3.2.1.21) into monomeric glucose units. Hemicellulases, such as xylanases and mannanases, hydrolyze the hemicellulosic polymers into its monomeric subunits xylose, mannose, galactose, rhamnose, arabinose, and glucose. Environmental degradation of lignocelluloses by higher fungi is an efficient but highly tedious process. Wood-feeding insects

(cf. below) depend on significantly faster metabolic processes. This raises much hope that the biochemical characteristics, especially the catalytic properties of insect enzymes, may speed up the unzipping of lignocelluloses in future biorefinery concepts.

3.2 Wood Pests

Several insects, such as bark and ambrosia beetles and termites, are able to feed on wood and thus are called xylophagous insects. Although they have been known as wood pests for centuries, their digestive systems still largely remain to be elucidated. In particular, the enzymatic apparatus needed for the oxidation of lignin and the hydrolysis of cellulose is only marginally understood. One reason might be the diverse sources of the key enzymes, which may be produced either by gut-inhabiting microorganisms [35], by symbiotic fungi cultivated by the insects [6], or by the insects themselves as endogenous enzymes [36]. Nevertheless, in recent years many efforts have been made to understand the lignocellulolytic system of the insects and the participation of their symbionts [7, 37, 38].

3.2.1 Hydrolytic Enzymes

Only recently, transcriptome studies revealed insights into active cellulase genes of insects [39–43]. Most prominent cellulases are endo-1,4-β-D-glucanases and β-D-glucosidases [44]. Several of these cellulases have been expressed in heterologous hosts and are biochemically characterized. An endo-1,4-β-D-glucanase from the lower termite *Reticulitermes flavipes* was expressed heterologously in a baculovirus expression system. The enzyme showed optimal activity against carboxymethyl cellulose (CMC) at pH 6.5–7.5 and 50–60 °C [45]. A recombinant β-glucosidase derived from the same organism exhibited the highest activity against cellobiose at neutral pH and demonstrated good stability up to temperatures of 40 °C. The enzyme's activity against laminaribiose (O-β-D-glucopyranosyl-1,3-D-glucose) was significantly lower [46]. Another β-glucosidase from the termite *Neotermis koshunensis* showed slightly higher activity against laminaribiose than against cellobiose, with an optimal temperature of 50 °C and an optimal pH of 5.0 [47]. The β-glucosidase of the higher termite *Nasutitermes takasagoensis*, expressed heterologously in *Pichia pastoris*, exhibited similar activities against cellobiose and laminaribiose, but it reached its maximum activity at 65 °C and pH 5.5 [48]. Most of the β-glucosidases were characterized by pH optima in the slightly acidic pH range of 5.0–6.0 (Table 2), although the pH in the termite gut varies between pH 6 and 10 [49, 50].

Some β-glucosidases from termites maintain their activity in the presence of high glucose concentrations, which is required for the saccharification of cellulose. The β-glucosidase of *N. takasagoensis* retained 50 % of its activity when incubated

Table 2 Insect derived β-glucosidases

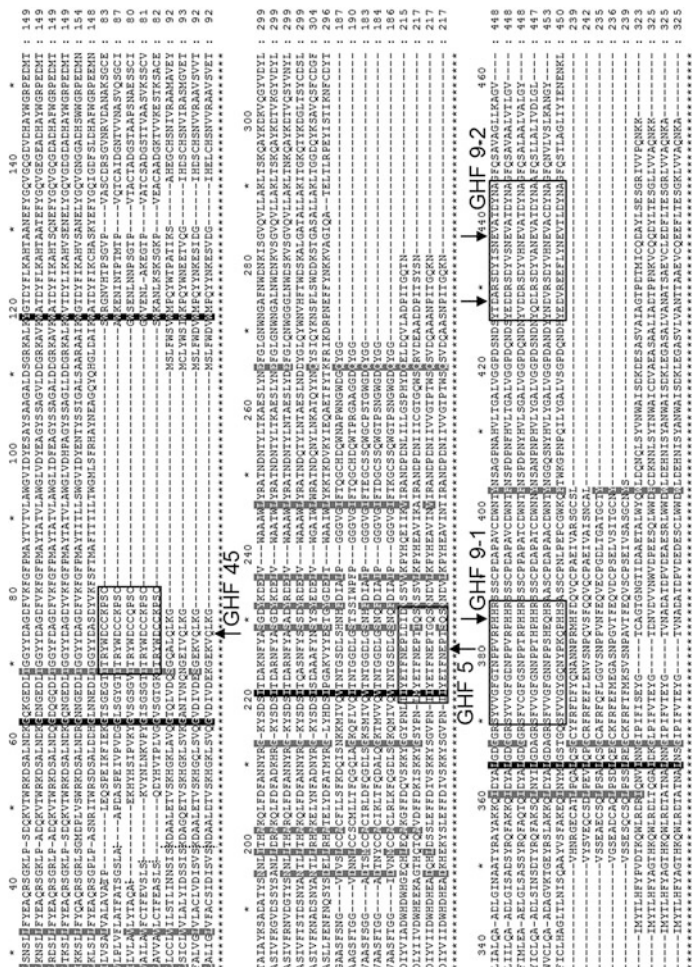
Organism	pH_{opt}	T_{opt} [°C]	Km	v_{max}	Reference
Termites					
Coptotermes formosanus	5.6-6.2	49	n.d.	n.d.	[51]
Macrotermes barneyi	5.0	50	n.d.	n.d.	[52]
Nasutitermes takasagoensis	5.5	65	n.d.	n.d.	[48]
Neotermis koshunensis	5.0	50	3.8 mM	220 μmol min^{-1} mg^{-1}	[47]
Neotermis koshunensis	5.0	50	0.77 mM[a]	16 μmol min^{-1} mg^{-1a}	[53]
Reticulitermes flavipes	7.0	n.d.	1.44 ± 0.14 mM	638.0 ± 39.0 μmol min^{-1} mg^{-1}	[46]
Beetles					
Bombyx mori	6.0	35	n.d.	n.d.	[54]
Rhynchophorus palmarum	5.0	50	0.31 mM	not given	[55]

[a] using p-nitrophenyl-β-D-*glucopyranoside* as a substrate, n.d. not determined

with 0.5 M glucose for 30 min [48]. The *Coptotermes formosanus* β-glucosidase retained approximately 90 % at 0.5 M glucose and 70 % at 1.0 M after 30 min [51]. The β-glucosidase of the termite *N. koshunensis* even showed a slight increase in its activity up to a glucose concentration of approximately 0.6 M [53]. Data on characterized beetle β-glucosidases are less prominent, but the so-far characterized β-glucosidases showed similar pH optima compared to the termite β-glucosidases (Table 2). Furthermore, the analyses of the transcriptomes of several beetles revealed the presence of cellulases [36, 40, 56], which should be characterized to evaluate their potential as future industrial biocatalysts.

Proteomic approaches disclosed the presence of hydrolytic enzymes in the Asian longhorned beetle *Anoplophora glabripennis*, such as β-glucosidases and carboxylesterases, which were identified by zymograms and subsequently sequenced by LC-electrospray-tandem mass spectrometry [57]. In this study, no β-1,4-D-endoglucanases could be identified, although the zymogram with incorporated CMC showed active bands. A similar approach was successfully applied for lignocellulose degrading enzymes isolated from the *A. glabripennis* symbiont *Fusarium solani* [58].

Further β-1,4-D-endoglucanases were cloned from the mulberry longhorn beetle *Apriona germari* [59, 60] and from the yellow-spotted longhorn beetle *Psacotea hilaris* [61]. The β-1,4-D-endoglucanases from *A. germari* and *P. hilaris* showed pH optima between 5.5 and 6.0. Termite β-1,4-D-endoglucanases showed similar characteristics, except for *R. flavipes,* which had an optimal pH of around 6.5–7.5 [45]. A much higher optimal pH of > 8.0 was observed for the β-1,4-D-endoglucanases of the red flour beetle *T. castaneum* [62]. Generally, the



β-1,4-D-endoglucanases found in insects can be divided into three glycoside hydrolase families (GHF) comprising specific patterns with different active side residues: GHF5—glutamic acid (E); GHF9—histidine (H), aspartic acid (D) and E; GHF45—D (Fig. 4).

◀ **Fig. 4** Alignment of amino acid sequences of various insect β-endoglucanases. All endo-1,4-β-D-glucanases comprised a signal peptide (dashed line) as analyzed by SignalP 4.1 [63]. Boxed sequences refer to glycoside hydrolase families 5, 9 (signature 1 and 2) and 45 (www.cazy.org). Arrows mark active side residues. Achi—*Anoplophora chinensis* (AFN89566); Ager—*Apriona germari* (AAU44973, AAR22385, AAX18655); Cfor—*Coptotermes formosanus* (ADB12483); Dvir—*Diabrotica virgifera virgifera* (JQ755253); Mdar—*Mastotermes darwiniensis* (CAD54728); Ntak—*Nasutitermes takasagoensis* (BAA33708); Oalb—*Oncideres albomarginata chamela* (ADI24131, ADI24132); Pcoc—*Phaedon cochleariae* (O97401); Phil—*Psacothea hilaris* (BAB86867); Rfla—*Reticulitermes flavipes* (AAU20853), Tcas—*Tribolium castaneum* (EFA05721); Temm—*Teleogryllus emma* (ABV32557). The integrated table classifies the orders [Isoptera (*black*), Orthoptera (*dark grey*), Coleoptera (*light grey*)] and the families [Ch—Chrysomelidae, Cr—Cerambycidae, Gr—Gryllidae, Ma—Mastotermidae, Tm—Termitidae, Tn—Tenebrionidae, Rh-Rhinotermitidae]

The phylogenetic analysis of aligned amino acid sequences of selected β-1,4-D-endoglucanases of termites and beetles shows an interesting pattern (Fig. 5). Three main clades reflect the classification of the β-1,4-D-endoglucanases in the different GHFs. All termite enzymes and the enzyme derived from the cricket *Teleogryllus emma* cluster together and comprise the GHF9 pattern (Fig. 4, black boxes GHF9-1 and GHF9-2, Fig. 5). All beetle (Coleoptera) enzymes except for that from *T. castaneum*, which is more related to the termite/cricket branch and also shows the GHF9 pattern, divide into two clades that belong to either GHF5 or to GHF45 (Fig. 5). Remarkably, the GHF5 β-1,4-D-endoglucanase of the white spotted longhorn beetle *Anoplophora chinensis* (synonym: *Anoplophora malasiaca*) shows activity not only on CMC but also on microcrystalline cellulose with comparable activities in the range of the well-known β-1,4-D-exoglucanase CBHI of *Trichoderma reesei* [64]. Probably more of these bifunctional β-glucanases are present in beetles, which helps to explain how these insects degrade cellulose containing substrates. Additionally, these bifunctional β-glucanases might help in enhancing the parallel saccharification of crystalline and amorphous cellulose.

In addition to the endogenous cellulolytic enzymes of beetles and termites, several studies focus on the hydrolytic activities of ants and their microbial community. De Fine Licht and colleagues [65] analyzed extracellular cellulolytic enzyme activities in ant-fungal gardens. Apart from cellulolytic activity, they determined peptidolytic and pectinolytic activity. Pectin-degrading enzymes, such as pectinases, pectin lyases and pectin esterases, represent a major class of enzymes present in fungal gardens and in ant fecal pellets [66, 67]. In a recent study, the decomposition of polysaccharides from plant cell walls was analyzed in fungal colonies of the leaf-cutting ant *Acromyrmex echinatior* [66]. In particular, pectin and hemicelluloses of the primary cell wall (xyloglycans) were degraded in the fungal garden, whereas cellulose remained more or less intact. These findings are in good agreement with data obtained by Rønhede et al. [67], who found four different pectin lyase isoforms and two pectin esterases in pure cultures of the fungal symbionts of *A. echinatior* and *Atta colombica*. In addition, they also detected CMC activity.

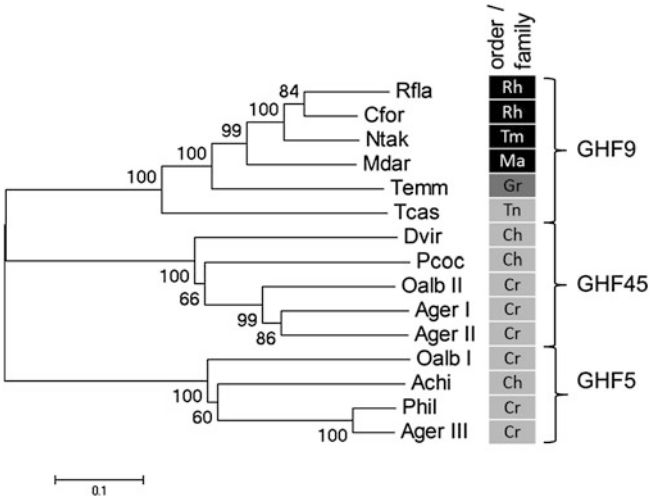


Fig. 5 Phylogenetic tree of aligned endo-1,4-β-D-glucanase deduced protein sequences calculated in the program MEGA by the neighbor-joining method using p-distance as an estimation model and pairwise deletion of the gaps. Bootstrapping was carried out with 500 replications. For species name abbreviations, see Fig. 4

3.2.2 Oxidative Enzymes

In a study performed by Geib et al. [37] the Asian longhorned beetle *A. glabripennis* and the Pacific dampwood termite *Zootermopsis angusticollis* were fed on oak or pine wood, respectively. Both insects could alter the chemical and physicochemical characteristics of hard- and softwood lignin dramatically. The authors observed side-chain oxidation, hydroxylation, and demethylation of the lignin monomers guaiacol and syringol. Surprisingly, the modification of the lignin occurred within hours, while moving through the insects' digestive system [37]. In a more recent study, the ascomycete *Fusarium solani*, isolated from larvae of *A. glabripennis*, was tested for its lignocellulolytic activities. Scully et al. [58] revealed manganese-independent peroxidase and laccase activity as well as cellulolytic and xylanase activities. In addition, multidimensional protein identification by LC–LC-MALDI-TOF–TOF analyses revealed the presence of supporting enzymes, such as esterases and hydrogen peroxide-producing enzymes [58].

In a study on the metagenome of the termite *Nasutitermes corniger* hindgut, no genes encoding lignolytic enzymes could be detected in this anaerobic microorganism community [68]. Most likely, lignin-degrading peroxidases (LiP, MnP, VP, or DyP) are produced by symbiotic organisms and—not like cellulolytic enzymes—by the insects themselves. On the other hand, laccases, which are also most prominent in filamentous fungi, do occur in insects [69, 70]. In insects, they are essential for the oxidation of catechols into quinones, which then undergo polymerisation with proteins to form the insect cuticle [69].

The knockdown of a laccase belonging to the "cuticular laccase group" (after Coy et al. [71]) in the pine sawyer *Monochamus alternatus* resulted in a thinner cuticle and a higher mortality of the insect [72]. Comparable results were also obtained for several stinkbugs [73]. In addition, laccases are also present in the gut system of insects, such as in the termite *R. flavipes*. Two laccases were identified, heterologously expressed, and characterized. Both laccases comprise all laccase-specific copper-binding sides (T1, T2, and T3) but showed no activity against the general laccase substrates ABTS and syringaldazine. Both laccases showed an increase in activity when hydrogen peroxide was added, revealing a peroxide-dependent phenol oxidase activity [71]. The potential applications of laccases are broad [74]. They may be employed for the production of middle-density fiber boards [75], for decolorization of dyes [76], or the clarification of juices [77]. Laccases originating from insect gut systems seem to have new properties compared to the fungal laccases [73]. In addition, laccases involved in sclerotization of the insect cuticle might find applications in the production of new polymers.

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Insect-Derived Chitinases

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Abstract Insect chitinases belong to family 18 of the glycoside hydrolase superfamily (GH18) and comprise endo-splitting enzymes that retain the anomeric β-(1,4) configuration of the cleavage products. However, some of them have lost their catalytic activity but retained the chitin binding activity and/or possess imaginal disc growth factor activity. In all sequenced insect genomes, multiple genes encode chitinases, which are differentially expressed during development and in various insect tissues. Some of them have nonredundant functions and are essential for growth and development. A characteristic property is their multidomain architecture, which comprises varying numbers of catalytic and chitin-binding domains that are connected by glycosylated serine/threonine linker regions. Based on sequence similarities and domain organization, they have been classified into eight different groups. Insect chitinases have gained increasing interest for use in the biological control of parasites, fungi, and insect pests, and some enzymes have properties that make them highly attractive for biotechnological applications.

Keywords Biopesticides · Carbohydrate binding module 14 · CBM14 · Chitin binding domain · Family 18 glycoside hydrolase · GH18 domain · Insect chitinase · Pest control

Abbreviations

Ac	Acetyl
CBM14	Carbohydrate binding module 14
GH18	Glycoside hydrolase family 18
20HE	20-hydroxyecdysone
IDGF	Imaginal disc growth factor
IPM	Integrated pest management
PM	Peritrophic matrix
STL	Serine/threonine-rich linker

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Contents

1 Introduction

Chitin is a β-(1,4)-linked polymer of *N*-aceytylglucosamine moieties, which is synthesized by a membrane-integral β-glycosyltransferase (chitin synthase; E.C. 2.4.1.16). It is secreted into the extracellular space, where it assembles into microfibrils [1]. Chitin is considered to be one of the most abundant macromolecules in the biosphere. The chitin microfibrils serve as structural scaffolds in cell walls, cuticles, shells, and intestinal peritrophic matrices (PMs). The capacity for chitin production is found in a vast variety of taxonomic groups including algae, fungi, protists, sponges, rotifers, nematodes, arthropods, cuttlefish, brachiopods, and mollusks (Table 1). Less known is the presence of chitin in tunicates and a few bony fishes. However, it seems that the ability to produce chitin has been lost at the root of the deuterostome lineage.

Chitin is particularly present in marine ecosystems because oceanic crustaceans produce most of its biomass (mainly pelagic zooplankton such as krill, which appears in gigantic swarms). In contrast to cellulose, whose biomass has been exploited since the early days of human history, chitin has remained an unused biomass resource for a long time. However, this has changed in recent years because it was recognized that chitin and its derivatives have unique physicochemical properties that allow a broad spectrum of technical applications [14]. More than 10,000 tons of chitin are extracted every year from crab and shrimp shells, which end up as waste in the seafood industry. Most of the chitin produced

Table 1 Localization and function of chitinous structures in various organisms

Organism	Localization	Biological function	Ref.
α-Protobacteria (Rhizobiales)	Extracellular space	Signaling molecules involved in nodulation of leguminous plants	[2]
Protozoa	Cyst wall	Physical and chemical resistance	[3]
Porifera	Sponge skeleton	Mechanical stabilization	[4, 5]
Fungi	Cell wall Yeast bud neck Spore wall Septa	Turgor resistance Stabilization of cell division zone Physical and chemical resistance separation of mother and daughter cells	[6, 7]
Nematodes	Pharynx Egg shell	Mechanical breakdown of food Physical and chemical resistance	[8]
Arthropods	Epidermal cuticles Shells Tracheal cuticle Peritrophic matrix Egg shells	Exoskeletal functions Protective functions Tracheal structure and development Mechanical protection, digestion, anti-infectious barrier Physical and chemical resistance, oogenesis	[1, 63]
Bivalves	Shell	Shell and nacre formation	[9]
Gastropods	Snail-shell, radula Peritrophic matrix	Shell formation, grazing Multiple protective functions	[10]
Cephalopods	Squid pen Cuttlefish bone Stomach cuticle	Endoskeleton Protective lining	[11]
Tunicates	Integument (test) Peritrophic matrix	Reinforcement Multiple protective functions	[12]
Teleost fish	Fin cuticle	Reinforcement	[13]

worldwide is used to obtain glucosamine and various oligosaccharides by acidic hydrolysis. Alkaline hydrolysis results in progressive deacetylation of chitin chains, eventually giving raise to chitosan, a polymer of β-(1,4)-linked glucosamines, which is a more soluble polymer. Chitosan is relatively nontoxic, has antimicrobial properties, and is a cationic polymer at acidic or neutral pH. Accordingly, it has manifold applications in industry. Chitosan-based materials are used as a preservative and dietary supplement in food industry, as a flocculation agent and for the adsorption of heavy metal ions in water treatment, and as antiseptic wound dressings and drug carriers in the pharmaceutical industry. Moreover, chitosan is subject of biopolymer research because the primary amino and the secondary hydroxyl groups are easy to modify. Many chitosan derivatives have been synthesized with the aim of developing intelligent biopolymers to fulfill specific functions. In contrast to cellulose, chitin is less stable in nature due to efficient decomposition. Accordingly, ancient chitin fossils are rare in contrast to cellulose fossils [15]. Biodegradation is mainly accomplished by hydrolytic enzymes called chitinases, which are found in a wide spectrum of organisms including viruses, archaea and eubacteria, protozoa, fungi, plants, and animals, independently of whether or not these organisms are capable of chitin synthesis.

In chitinolytic bacteria that do not synthesize chitin or chitooligomers themselves, chitinases are produced in the course of nutrition, enabling them to use chitinous material as a carbon and nitrogen source. Specifically, species of the genus *Streptomyces* transform insoluble chitin into soluble, metabolizable compounds by means of different chitinolytic enzymes and chitin-binding proteins that mediate adherence to chitinous substrates [16]. In plants, chitinases are thought to be involved in the defense against fungal pathogens, but some chitinase-like proteins lack antifungal activities and seem to play roles during development [17]. In carnivorous plants, they have been detected in the pitcher fluid where they appear to be involved in inducing the trap digesting machinery, in the defense against pathogens, and in chitin decomposition [18, 19]. In chitin-less mammals, chitinases and chitinase-like proteins have been shown to modulate immune responses (reviewed recently by [20]). In all chitin-producing organisms, however, chitinolytic enzymes are essential for maintaining normal lifecycle functions, such as cell division and sporulation in fungi or morphogenesis and metamorphosis in arthropods [21].

Chitinases from various sources have been attracting interest for biotechnological applications in the chemical and pharmaceutical industry because they can convert chitinous material from natural sources (such as crab shells) into usable components. Recombinant insect-derived chitinases may serve as powerful enzymes in such catalytic systems. In addition, chitinases and their inhibitors possess high potential as fungicides for the treatment of mycoses in animal and humans, therapeutic compounds against parasites, and biopesticides for the control of insect pests. This review focuses on insect-derived chitinases and discusses their potential in insect biotechnology (referred to as *yellow biotechnology* in this book).

2 Molecular Properties of Insect Chitinases

Biodegradation of chitin is accomplished by different types of enzymes that include chitinases (EC 3.2.1.14) and *β-N*-acetylhexosaminidases (EC 3.2.1.52), with the latter enzymes acting on chitooligosaccharides that were generated by chitinases cleaving longer chitin chains. Chitinases have been classified into two families of glycoside hydrolases, GH18 and GH19 (http://www.cazy.org; [22]). Although GH18 chitinases are widely distributed in all kingdoms, including insects, GH19 chitinases are restricted to plants, except for a few enzymes reported from viruses, bacteria, nematodes, and arachnids. GH18 and GH19 chitinases show only limited sequence similarities and differ in their catalytic mechanisms. GH18 chitinases and chitinase-like proteins are frequently endo-splitting enzymes but also contain catalytically inactive proteins such as imaginal disc growth factors (IDGFs), stabilin-1 interacting chitinase-like proteins (SI-CLPs), endo-*β-N*-acetylglucosaminidases (ENGases) and chitolectins [23]. The GH18 chitinases perform substrate-assisted catalysis involving a oxazolinium ion intermediate state [24]. This reaction results in the retention of the *β*-configuration at the anomeric

carbon of the cleavage product. In contrast, GH19 chitinases operate by an acidic catalytic mechanism through an oxocarbenium glycosyl-enzyme intermediate, resulting in the inversion of the anomeric carbon atom (i.e., α-anomeric configuration). The soluble products of the reaction catalyzed by chitinases are small chitooligosaccharides (predominantely chitobiose and chitotriose), which become substrates for exo-splitting *β-N*-acetylhexosaminidases (family GH20) and remove terminal nonreducing GlcNAc residues. GH18 and GH20 chitinioyltic enzymes frequently act in concert to facilitate chitin degradation.

2.1 Domain Architecture of Insect Chitinases

In insects, chitinases belong exclusively to family GH18 and exhibit mostly endo-splitting activity. They are presumably present in all insect orders. GH18 proteins have been reported in various dipteran, leipidoperan, coleopteran, hymenopteran, and hemipteran species, where they primarily function in remodeling chitinous structures, innate immunity, and development (see below). They exhibit a modular architecture composed of catalytic domains (GH18 domains), cysteine-rich chitin-binding domains (CBM14 or peritrophin A domains) and serine/threonine-rich linker domains (STL).

Insect chitinases have been particularly diversified during evolution, giving rise to eight different phylogenetic groups, which differ in their primary structures and domain architectures (Table 2, [25]). This classification was mainly based on genome-derived amino acid sequences and domain architectures for GH18 protein from the malaria mosquito *Anopheles gambiae*, the fruit fly *Drosophila melanogaster*, and the red flour beetle *Tribolium castaneum*. However, it has to be noted that a more recent study on GH18 proteins encoded in the genome of the pea aphid *Acyrthosiphon pisum* has revised this classification to some extent; this phylogenetic analysis, which included GH18 sequences from more insect species, did not well support groups IV, VI, and VII [26]. This finding is also reflected by the low bootstrap values at the corresponding branches in a phylogenetic tree of GH18 proteins from *A. gambiae*, the honey bee *Apis mellifera*, the silkmoth *Bombyx mori*, *D. melanogaster,* and *T. castaneum* (Fig. 1). Nevertheless, the old classification is used in this review article because most of the functional studies refer to this classification.

Group I chitinases are composed of a signal peptide, and one each of the GH18, STL and CBM14 domains. Interestingly, group I chitinase genes have expanded specifically in mosquitoes by gene duplication events [27]. Group II chitinases are significantly larger than group I chitinases because they are composed of a signal peptide followed by 4–5 GH18, multiple STL, and 4–7 CBM14 domains. Except for dipteran species, which have only 4 GH18 and 4 CBM14 domains, these domains are typically arranged as follows: GH18-CBM14-GH18-$(CBM14)_3$-$(GH18)_2$-CBM14-GH18. By contrast, dipteran group II chitinases are arranged like this: GH18-$(CBM14)_3$-$(GH18)_2$-CBM14-GH18. Some of the GH18 domains

Table 2 Domain architecture of group I-VIII insect chitinases

Group	SP[a]/TMH	GH18	CBM14	STL
I	SP	1	1	1
II	SP	4–5	4–7	Multiple
III	TMH	2	1	0
IV	SP	1	0 (1)	0
V	SP	1[b]	0	0
VI	SP	1	1	1[c]
VII	SP	1	0	1
VIII	TMH	1	0	0

[a] SP, N-terminal signal peptide; TMH, N-terminal transmembrane helix; GH18, catalytic domain similar to family 18 glycoside hydrolases; CBM14, chitin-binding domain belonging to family 14 of carbohydrate-binding modules; STL, serine/threonine-rich linker
[b] GH18 domain catalytically inactive
[c] Very large C-terminal serine/threonine-rich region

(in particular the second GH18 domain) appear to be catalytically inactive because a critical glutamate, which acts as a proton donor in the reaction cycle, is replaced by a nonacidic amino acid [28]. Group III chitinases are typically made up of a single *N*-terminal transmembrane helix (TMH), two adjacent GH18 domains, and one *C*-terminal CBM14 domain. From sequence alignments, it was concluded that the catalytic GH18 domains may have different functions and/or origins. The prediction of an *N*-terminal TMH region suggests that group III chitinases are membrane anchored. This assumption has been supported by analyzing Hi-5 cells expressing TcCHT7 from *T. castaneum*. The enzyme was apparently anchored by the TMH to the surface of the insect cells with the catalytic GH18 domains facing the extracellular space, as revealed by their ability to hydrolyze chitin without lysing the cells [29]. Group IV chitinases constitute a highly divergent group. They are usually encoded by multiple genes in a single insect species. Group IV chitinases are composed of a *N*-terminal signal peptide followed by a GH18 domain. Many of these chitinases (but not all) lack a CBM14 domain. Specifically, *T. castaneum* group IV chitinase genes have expanded in two separate monophyletic clades that consist of five and nine genes [26]. Group V chitinases always lack CBM14 domains, but possess an *N*-terminal signal peptide and a GH18 domain, which exhibits amino acid substitutions known to abrogate catalytic activity [30]. This group includes the imaginal disk growth factors (IDGFs), which are required for the proliferation, polarization, and motility of imaginal disc cells [31]. Group VI chitinases are similar to Group I chitinases. However, the *C*-terminal serine/threonine-rich region, which seems to be heavily glycosylated, largely extends the molecular mass of this protein. Group VII chitinases in turn resemble group IV chitinases in overall structure, but phylogenetic analysis revealed that this group is an outlier of group II chitinases. They have an N-terminal signal peptide and a GH18 domain, but they are devoid of a CBM14 domain. Group VIII chitinases have a GH18 domain but lack a signal peptide and a CBM14 domain. Like in group III chitinases, to which they are phylogenetically

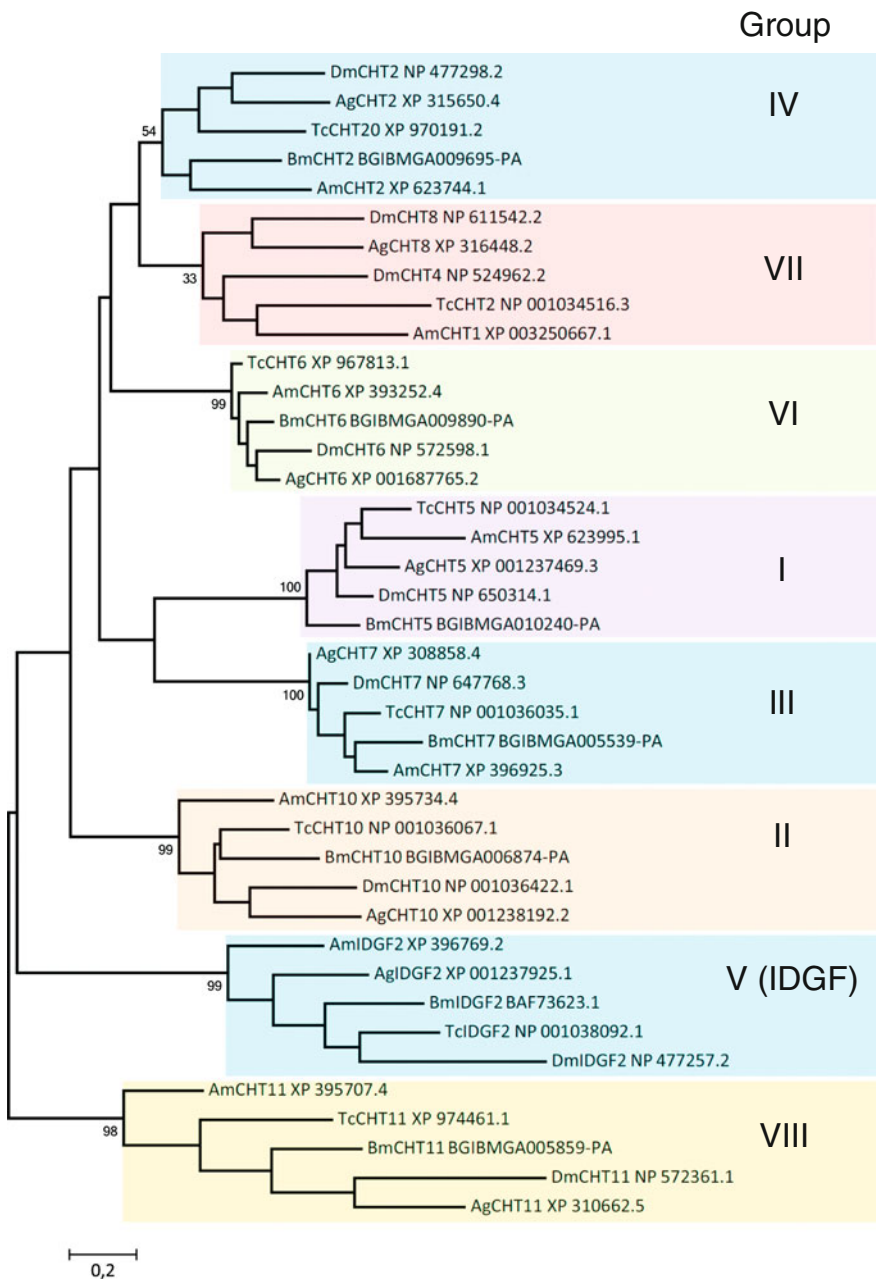

Fig. 1 Phylogenetic tree for various GH18 chitinases and chitinase-like proteins. The maximum likelihood tree was calculated on the basis of a ClustalW alignment (Blosum62) of amino acid sequences for chitinases and chitinase-like proteins. The scale bar indicates an evolutionary distance of 0.5 amino acid substitutions per site. Bootstrap values are given in percentages at the internodes. Different groups of GH18 chitinases are indicated by colored shadings. Am, *Apis mellifera*; Ag, *Anopheles gambiae*; Bm, *Bombyx mori*; Dm, *Drosophila melanogaster*; Tc, *Tribolium castaneum*. Accession numbers are given after the species abbreviation

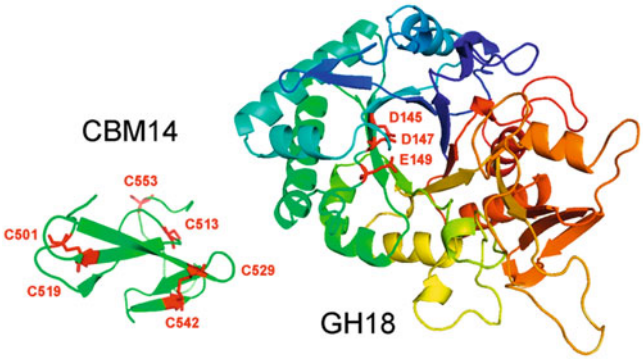

Fig. 2 Homology-based models of the GH18 and the CBM14 domains of a group I chitinase from *Anopheles gambiae* (XP_001237469.3). The positions of catalytic site residues of the GH18 domain and conserved cysteines of the CBM14 domain are indicated with red colors. The serine/threonine rich region linking the GH18 and CBM14 domains has not been modeled and is not included. The (α/β)8 barrel (TIM barrel) is depicted in the center of the GH18 domain

most closely related, the GH18 domain is preceded by an *N*-terminal TMH. This suggests that these chitinases are also membrane-bound.

2.2 The Catalytic GH18 Domain

The crystal structures for several bacterial, fungal, plant, and mammalian GH18 chitinases have been determined [32–35]. The structural hallmark of the GH18 domain is a (β/α)8 barrel (TIM barrel) fold, which is also illustrated in the center of a homology-based structural model for the group I chitinase from the malaria vector *A. gambiae* (Fig. 2). Some of the loops extending from the TIM barrel are thought to form a narrow but long substrate-binding cleft, which allows binding of at least five sugar units. In comparison, plant GH19 have a shorter and wider substrate binding site, which is predicted to allow binding of only three sugar units [36]. So far, only one crystal structure of an insect GH18 protein has been determined. Specifically, the structure of the imaginal disk growth factor (IDGF2) from *D. melanogaster* has been solved at a resolution of 1.3 Å [37]. IDGF2 has a classical TIM barrel of GH18 chitinases, however, with two prominent insertions. One insertion is highly conserved in group V chitinases (see below), and resides between the β-4 strand and the α-4 helix, a surface region that is exposed to the solvent. The other localizes between the β-7 strand and the α-7 helix and forms an additional $\alpha + \beta$ domain, which is present in all insect chitinases, although it is highly variable in sequence. Although the first insertion has been associated with proteolytic degradation, the second insertion may determine the cleavage modus of insect chitinases (whether they are endo- or exosplitting or processive enzymes). IDGF2 is catalytically inactive because amino acid substitutions in the conserved

GH18 domain appear to preclude chitin hydrolysis [38]. This is particularly due to the exchange of the catalytic glutamate by a glutamine, which abolishes hydrolytic activity in chitinases (see below).

The GH18 domain comprises four signature sequences, which are highly conserved in insect chitinases: motifs I-IV reside in the β-strand 3, 4, 6, and 8, respectively, and have the consensus sequences K(F/V)M(V/L/I)AVGGW, FDG(L/F)DLDWE(Y/F)P, M(S/T)YDL(R/H)G, and GAM(T/V)WA(I/L)D [25]. Site-directed mutagenesis performed with a recombinant group I chitinase from *Manduca sexta* has established the roles of three acidic amino acids in motif II (D142, D144, and E146, underlined above). The corresponding amino acids D145, D147, and E149 are also shown in the structural model for the group I chitinase of *A. gambiae* (Fig. 2). Although none of these amino acids were required for chitin binding in *M. sexta*, they were more or less critical for catalysis [28]. The glutamate E146 was most important for catalytic activity; its substitution by glutamine or even by the negatively charged aspartate led to a complete loss of enzymatic activity. This finding suggests that E146 acts as an acid/base catalyst in this reaction. The aspartates D142 and D144 are less critical; they seem to function in determining the pKa values of the other two residues and stabilizing the transition state, respectively. Similar to D142, tryptophan W145 within motif II appears to be necessary for optimal enzyme activity but is not required for chitin binding [39].

2.3 The Chitin-Binding CBM14 Domain

The CBM14 modules of chitinases are widely distributed chitin-binding domains, which are also found in insect PM proteins (PMPs) and cuticle proteins analogous to peritrophins (CPAPs) [40]. The chitin-binding function of the CBM14 has been shown in several cases [41]. The only structure available for an invertebrate CBM14 motif is that for tachycitin, a 73-residue polypeptide with antimicrobial activity from hemocytes of the horseshoe crab (*Tachypleus tridentatus*). Nuclear magnetic resonance spectrometry revealed that the polypeptide consists of an *N*-terminal part made of a three-stranded β-sheet and *C*-terminal part made of a two-stranded β-sheet following a short helical turn [41]. Five disulfide bridges formed between 10 cysteines stabilize this β-sandwich structure. Some of the β-sheets form a hydrophobic binding pocket, which involves conserved polar and hydrophobic amino acid residues [42]. In contrast, insect CBM14 domains have only six conserved cysteines, presumably forming three disulfide bridges. The characteristic spacing between these cysteines is $CX_{13-20}CX_5CX_{9-19}CX_{10-14}CX_{4-14}C$ [40, 43]. Figure 2 shows a structural model of the CBM14 domain from a group I chitinase of *A. gambiae*, highlighting these conserved cysteines. The CBM14 domain is thought to enhance the affinity of the chitinase for its polymeric insoluble substrate, so that these enzymes are more effective on chitin than on chitooligosaccharides [44].

2.4 The Serine/Threonine-Rich Linker Region

The GH18 and CBM14 domains are frequently but not always connected by serine/threonine-rich linker regions (STL), which are presumably modified by mucin-type O-glycosylation. This may affect protein stability, particularly in protease-rich environments, such as the molting fluid or the gut lumen, where insect chitinases frequently are operating [44]. The first step of the mucin-type O-glycosylation is catalyzed by a polypeptide *N*-acetylgalactosaminyl transferase (GalNAc transferase). This enzyme transfers the sugar moiety from UDP-GalNAc to the serine and/or threonine residues of the acceptor polypeptide. Interestingly, GalNAc-transferases are absent in plants but present in insects, such as *D. melanogaster*, where specific isoforms appear to have unique functions in particular tissues, including epithelia known to synthesize and secrete chitinases [45]. The STL region is predicted to be an unfolded polypeptide. Because it is therefore not possible to model the structure of this region, the GH18 and CBM14 of the *A. gambiae* chitinase domains were drawn separately in Fig. 2.

2.5 Enzymatic Properties of Insect Chitinases

In a comprehensive enzymatic study, Zhu et al. compared the properties of group I, IV, or V chitinases from *T. castaneum*, *D. melanogaster,* and *M. sexta*. They expressed the chitinases in Hi5 cells in insect cells using a baculoviral system and purified them by Ni–NTA or DEAE-Sepharose chromatography [38]. Subsequently, the enzymatic and chitin-binding properties were compared. Except for group V proteins, which comprise the group V IDGFs, all chitinases exhibited chitinolytic activities. However, the enzymes from different groups differed with respect to their ability to bind chitin, immunological cross-reactivity, kinetic properties, pH dependency of activity, and their preference for oligomeric or polymeric substrates. Group I chitinases cleaved both polymeric and oligomeric substrates, whereas group IV chitinases exhibited no and only little chitinolytic activity for oligomeric substrates. The tested group I chitinases showed two optima at approximately pH 6 and 9, whereas group IV chitinase either had only a single optimum at pH 6 or were highly active over a broad pH range from pH 4 to 9. All tested chitinases and chitinase-like proteins, including the group V IDGFs, bound tightly to colloidal chitin.

All catalytically active insect GH18 chitinases studied so far have been shown to be endo-splitting enzymes that cleave chitin or chitooligosaccharides comprising at least three sugar moieties. The recombinant 65-kDa chitinase from *Bombyx mori* preferentially cleaves the β-(1,4) glycosidic linkage of GlcNAc oligosaccharides after the second position from the nonreducing end, retaining the β-anomeric configuration of the product [46]. In contrast, the chitinase from *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), which may

have a bacterial origin but is expressed in virus-infected insect cells, exhibits both endo- and exo-splitting activities [47]. However, as in the case of insect chitinases, cleavage of olilgosaccharides occurs at the linkage between the second and third sugar moiety from the nonreducing end [48]. The catalytic mechanism of insect chitinases has not been explained in detail, but it is likely that—like other non-insect GH18 chitinases—they act through substrate-assisted catalysis involving an oxazolinium intermediate state, as described above.

Besides their chitinolytic activity, many bacterial, fungal, plant, and mammalian GH18 chitinase possess transglycosylation activity, which allows the formation of new glycosidic bonds between donor and acceptor saccharides. In retaining glycoside hydrolases, the transglycosylation reaction occurs through a double-displacement mechanism using another acceptor molecule (carbohydrate or an alcohol) instead of water [49]. This type of reaction leads to the interconversion of oligosaccharides of different compositions and lengths. Oligosaccharides of specific compositions and length, however, are attracting increasing interest in the food and pharmaceutical industries due to their potential use as therapeutics, prebiotics, or in plant protection [50–53]. Therefore, transglycosylation is considered to have a high potential in generating defined oligosaccharides because their chemical synthesis is highly challenging. The development of methods for enzymatic synthesis of oligosaccharides may include insect-derived GH 18 chitinases because it is likely that many of these enzymes exhibit transglycosylation activity.

2.6 Insect Chitinase Gene Expression During Development

In insects, chitinase encoding genes are differentially expressed during development, and the expression of some genes is restricted to certain tissues. The expression profiles have been determined in detail by reverse-transcription and qualitative polymerase chain reaction for *T. castaneum*, *A. gambiae,* and *A. pisum* [26, 54, 55].

In *T. castaneum*, the genome harbors in total 22 genes encoding chitinases and chitinase-like proteins [55]. The group I, II, III, and V genes (*TcCHT5*, *TcCHT10*, *TcCHT7,* and *TcIDGF2/4*, respectively) were found to be expressed throughout all stages of development. Transcripts for the group VI gene *TcCHT6* were detected in all stages except for adults, and transcripts for the group VII gene *TcCHT2* were found in all stages except for embryos and adults. The group VII gene *TcCHT11* was found to be expressed in all but penultimate instar larvae. Transcripts for all 14 group IV genes (*TcCHT4*, *−8*, *−9*, *−12* to *−22*) were detectable in the feeding stages (larvae and adults). However, expression of two of these genes (*TcCHT4* and *TcCHT16*) was also found at lower levels in pharate pupal and pupal stages. Moreover, the group IV genes (except for *TcCHT4*) were found to be expressed exclusively in the larval midgut, and gene expression varied in different gut

regions. The latter finding suggests that group IV chitinases act on the chitin network of the PM and/or dietary components.

Overall, there are similarities and differences between the expression profiles of *T. castaneum and A. gambiae,* the latter of which has 20 chitinase genes [54]. Expression of group I, II, III, and V genes (*AgCHT5−1 to−5*, *AgCHT10*, *AgCHT7* and *AgIDGF2/4*, respectively) was detectable in all developmental stages at various levels, from embryos (except for *AgCHT10*) to adults. The two IDGF genes (*AgIDGF2* and *−4*) were constitutively expressed at higher levels. The expression of the eight group IV genes of *A. gambiae* (*AgCHT4*, *−8*, *−9*, *−12*, *−13*, *−16*, *−23*, *−24*) showed various levels of expression in the different developmental stages tested. Notably, the group IV chitinases *AgCHT12*, *AgCHT13* and *AgCHT23* were almost exclusively expressed in the larval stages, whereas *AgCHT8* was expressed in pupae and adults. All group IV genes were detected in the gut, either in the foregut (*AgCHT4*, *−12*, *−16*, *−23*), midgut (*AgCHT9*, −13, −16, −23), or hindgut (*AgCHT9, −16, −23*), except for *AgCHT24*, which was not tested for its expression in the gut. The group VII gene was also found to be specifically expressed in the *AgCHT4* in the foregut.

In *A. pisum,* the expression of *ApCHT2 (*group IV), *−3* (group VI), *−4* (group III), and *−8* (unclassified) was found to be highly upregulated in embryonic tissues [26], and that of two distinct chitinase-like genes, *ApCHT6* (group IV) and *ApENGase* (ENGase), was significantly higher in the midgut than in other tissues (classification according to [25]).

Upregulation of the expression of chitinase-encoding genes involves transcriptional control mechanisms mediated by molting hormones. The injection of 20-hydroxyecdysone (20HE) into ligated larvae of *M. sexta* and *B. mori* stimulates the activity of chitinases in the integument [56, 57]. This increase in chitinolytic activity correlates with raised transcript levels that have been measured for group I chitinase genes from *M. sexta* following 20HE injection [58]. The 20HE-induced increase of transcript levels was suppressed when fenoxycarb, a juvenile hormone mimic, was topically applied. Similar results have been reported for chitinases from the silkworm *B. mori* [59], the spruce budworm *Choristoneura fumiferana* [60], the mealworm beetle *Tenebrio molitor* [61], and even from crustaceans such as the Chinese shrimp *Fenneropenaeus chinensis* [62]. Thus, 20HE-dependent control of chitinase gene expression and chitinolytic activity appears to be widely distributed among insects and possibly also other arthropods. Whether gene activities of chitinase-encoding genes are directly or indirectly controlled has not been elucidated yet.

2.7 Functions of Chitinases in Insects

The finding that gene expression of chitinases and chitinase-like proteins varies between different developmental stages and tissues indicates that chitinases have distinct functions. This conclusion was supported by an exciting study in *T.*

castaneum using systemic RNA interference to systematically knockdown transcripts for group I (*TcCHT5*), II (*TcCHT10*), III (*TcCHT7*), IV (*TcCHT8*, *−14*, *−16*), V (*TcIDGF2* and *−4*), VI (*TcCHT6*), and VII (*TcCHT2*) chitinase or chitinase-like protein encoding genes [55]. Injection of dsRNA specific for *TcCHT5* resulted in molting defects and death only during pupal-adult molting, whereas that for *TcCHT10* caused arrest at all larval–larval, larval–pupal, and pupal–adult moltings as well as defects in egg hatching. When penultimate instar larvae were injected with dsRNA specific for *TcCHT7*, they completed larval–larval and larval–pupal molts, but the pupae failed to contract their abdomens and to fully expand their elytra. Injection of dsRNA for *TcIDGF4* into penultimate or last instar larvae resulted in death of the insects during adult eclosion. The injection of dsRNAs for the genes *TcCHT2*, *TcCHT6*, *TcCHT8*, *TcCHT14*, *TcCHT16,* and *TcIDGF2* failed to result in observable phenotypes.

Chitinase and chitinase-like proteins are thought to have important functions during the growth and development of insects [21, 25]. During molting, they assist in the degradation of inner parts of the chitinous endocuticle, a process required to shed off the old cuticle (exuvia). In the intestinal tract, they have important roles in digestion of chitin-containing food (including exuvia, which is frequently eaten after molting) and in the turnover of the chitin-containing PM, which lines the midgut epithelium. It protects the midgut epithelium from abrasive particles, divides the gut lumen into distinct digestive compartments, and acts as an anti-infectious barrier [63]. In addition, chitinase-like proteins promote cell proliferation and have functions in the innate immune system. From the expression profiles and the RNAi studies, several important conclusions on the functions of chitinases in insects can be drawn.

Group I–II chitinases act in the degradation of the endocuticle during molting with activities that have different effects on larval–larval, larval–pupal, and pupal–adult molts. Partially, these chitinases may have redundant functions. They are secreted into the molting fluid, which is known to contain proetolytic and chitinolytic activities, and degrade the inner parts of the old cuticle to allow molting [64]. Interestingly, the chitinases appear not to be excluded from the newly synthesized cuticle by an impermeable layer (also called cuticulin or envelope) as previously assumed, because a recent study performed in *T. castaneum* provided evidence that the new cuticle is protected from chitinolytic activity by a cuticle-organizing protein named after the *Drosophila* mutant Knickkopf [65].

Group III chitinases differ from group I and II chitinases in that they are anchored to the plasma membrane by an *N*-terminal transmembrane helix. They appear not to be required for molting but for processes that occur immediately after pupation such as abdominal contraction and the extension of wings and elytra. How they accomplish these function is not known.

Group IV chitinases appear to have functions in the intestinal system because they are only expressed in different parts of the gut. This suggests that they are involved in the degradation of chitinous material either assimilated with food or as part of the PM. Some of these gut-specific chitinases may also have immune functions. For example, the group IV chitinase GmCHT1 from the tsetse fly

Glossina morsitans morsitans contains a GH18 domain and a CBM14 domain but lacks an STL region. The *GmChT1* gene is expressed in the fat body and in the milk glands of pregnant female flies. The tsetse flies undergo viviparous reproduction and the chitinase becomes transmitted to the larvae by feeding of a milk-like substance secreted in the mother's uterus. Once taken up by the larvae, it may have immunological function in the larval gut, protecting against fungal pathogens [66].

Groups V chitinase-like proteins (IDGFs) are predominantly expressed in embryonic yolk cells and fat body; they may act as chitolectins binding to cell surface receptors. They have been shown to promote cell lineages derived from *Drosophila* imaginal discs in cooperation with insulin-like growth factor [31]. In hemocyte and fat body cell lines derived from the cabbage armyworm, *Mamestra brassicae*, however, they stimulated cell proliferation independently from the presence of insulin-like peptides [67]. Next to their function in cell proliferation, some IDGFs also have immune functions. In *A. gambiae* two group V proteins, AgBr1 and AgBr2 are secreted into the hemolymph after challenging the mosquitoes by bacterial infections. AgBr1 and AgBr2 are proteolytically processed after exposure to bacteria or peptidoglycans, as reported for some other IDGFs [68]. Finally, honeybee salivary glands secrete IDGF4 into the royal jelly and honey. This finding implies that IDGF4 might affect growth, physiology, or even behavior of other bees in the hive [69]. In contrast to group I–V chitinases, the functions of group VI–VIII chitinases have not been addressed so far.

3 Chitinase Inhibitors in Human Health and Pest Control

3.1 Pseudosugars, Cyclic Peptides, and Purine Derivatives

Inhibitors of GH18 chitinases demonstrate significant biological activities against insect pests, fungi, and protozoan/nematodal parasites, as they interfere with essential physiological functions. Among the most potent natural inhibitors of chitinases are allosamidin, argifin, and argadin [70]. Allosamidin is one of the best characterized inhibitors. It was isolated from *Streptomyces* species and exhibits an inhibitory activity against GH18 chitinases, while being inactive on GH19 chitinases. It is a pseudosugar (pseudotrisaccharide) consisting of two units *N*-acetyl-D-allosamine and one unit of an aminocyclitol derivative (Fig. 3). Allosamidin is thought to specifically block the transition state of GH18 chitinases. The compound has been shown to inhibit chitinases from various insects, including the silk moth *B. mori*, the nonbiting midge *Chironomus tentans,* and the green peach aphid *Myzus persicae* [71–73]. When applied to larvae of *B. mori*, *Leucania separata* (common armyworm), *Tineola bisselliella* (webbing cloth moth), or *Lucilia cuprina* (Australian sheep blowfly), it inhibited larval to pupal molt [74, 75]. Feeding *Phlebotomus papatasi* sandflies, a vector for the *Leishmania* parasite, with allosamidin supplemented blood led to a thickening of the PM from the

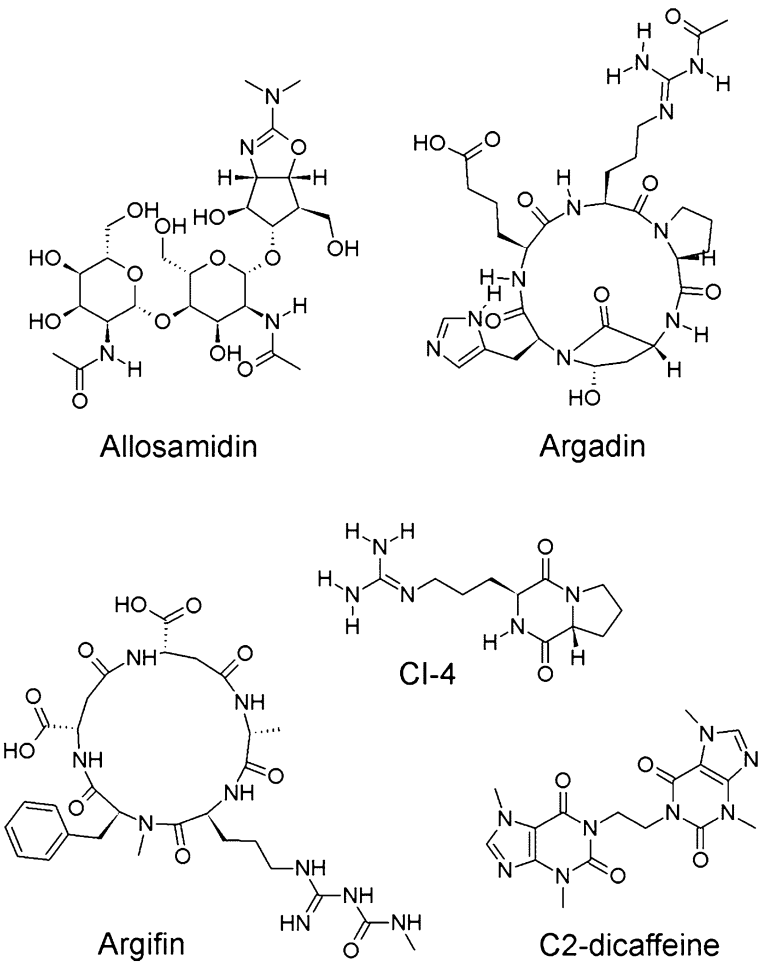

Fig. 3 Chemical structures of GH18 chitinase inhibitors. The depicted inhibitor structures include the pseudotrisaccharide allosamidin, the cyclopentapeptides argadin and argifin, the small cylic peptide CI-4, and the purine-derivate C2-dicaffeine. Chemical structures were obtained from the Binding database and visualized with ACD ChemSketch 8.0

midgut. It also prevented early parasite mortality seen in infected flies, suggesting an important role of the PM as an anti-infectious barrier [76].

Similar results were obtained when allosamidin-supplemented blood was fed to female yellow fever mosquitoes [77]. This treatment resulted in the formation of an atypical thick PM, whereas the addition of exogenous chitinase completely blocked PM formation. Most notably, allosamidin inhibits the transmission of the *Plasmodium* ookinetes by blocking the parasite's chitinase, which facilitates penetration of the mosquito's PM [78, 79]. Although allosamidin has evidently a high potential as an active ingredient of antiparasite drugs and insecticides, its total

synthesis is complex and expensive, thus currently preventing its use in parasite and pest control regimes [70]. The synthesis of allosamidin analogs provided insights into the structural requirements for chitinase inhibition. Blattner et al. evaluated different allosamidin analogues to provide insights into structure–activity relationships [75, 80]. For a chitinase from *Chironomus tentans*, the removal of a single *N*-acetylallosamine residue did not impair inhibitory activity and glucosamine can be exchanged against allosamine without any negative effect [80]. Similar results were obtained when testing the insecticidal activity for *Tineola bisselliella* and *Lucilia cuprina* larvae [75]. The authors came to the important conclusion that the β-(1,4) linkage between the sugar or pseudosugar moieties is necessary for chitinase inhibition and insecticidal activity. Thus, β-(1,4)-gluco disaccharides are attractive candidate molecules for chitinase inhibition. Based on these findings, Dusssouy et al. reported that GlcNAc-β-(1,4)Glc disaccharides containing 2-O-acetyl and/or 6-sulfate groups are highly potent inhibitors of insect chitinase activity and that they have strong aphidicidal activities when tested on hemipteran *Myzus persicae* [81]. However, the latter effects could not be explained exclusively on the basis of chitinase inhibition.

Argifin and argadin are an alternative class of GH18 chitinase inhibitors. Their chemistry is not based on sugars, and their synthesis is less challenging. These molecules are cyclopentapeptides (Fig. 3), which were isolated from mycelia of *Gliocladium* and *Clonostachys* species, respectively [82, 83]. It has to be noted, however, that the taxonomic classification of these fungi is uncertain and needs to be reassessed using appropriate DNA markers [84]. Both inhibitors mimic the interactions of GH18 chitinases with chitooligosaccharides and interact with side chains D142, E144, and Y214 in the active site required for catalytic activity [85]. Argadin more strongly inhibits GH18 chitinases than allosamidin does, whereas argifin exhibits weaker inhibition due to structural differences between the two peptide backbones. The detailed structural information available for these types of inhibitors allowed identification of a tiny nine-atom active fragment of argifin, which is a micromolar inhibitor of GH18 chitinase from *Aspergillus fumigatus* [86]. Another peptide-based inhibitor is the small cyclic peptide CI-4 (cyclo (L-Arg-D-Pro)) (Fig. 3), which in contrast to the cyclopentapeptides blocks chitinase activity by structurally mimicking the reaction intermediate [87]. It was first isolated from the marine *Pseudomonas* species [88]. A screening of a compound library of 880 drug molecules identified recently three xanthine derivatives, theophylline, caffeine, and pentoxifylline as moderate inhibitors of bacterial, fungal, and human family 18 chitinases [89]. Moreover, a fragment-based, computer-aided approach to screen commercially available chemical structures allowed these investigators to identify a xanthine-derivative (C2-dicaffeine) as GH18 chitinase inhibitor that acts in the low micromolar range [90]. Some of the latter chitinase inhibitors may also be active on insect-derived enzymes.

3.2 Antibodies to Chitinases Block Parasite Transmission

Malaria is one of the most important parasitic diseases, affecting hundreds of millions of humans per year, with at least 1 million deaths. The parasite is transmitted by female *A. gambiae* mosquitos that inject infectious sporozoites while taking a blood meal. Before the mosquitoes can transmit the parasite, they have to get infected themselves by ingesting male and female gametocytes from a blood meal of a malaria-infected person. After mating, the resulting zygote elongates into the invasive motile form, which is called ookinete. The ookinete must penetrate the PM before it can invade the midgut epithelium to reach the hemolymph site, where it eventually develops into sporozoite-forming oocysts [91]. The ookinete produces a GH18 chitinase that facilitates traversal of the chitin-containing PM [92]. Both the targeted disruption of the *Plasmodium* chitinase-encoding genes and the inhibition of its chitinolytic activity by allosamidin led to a significant reduction of infectivity in *A. gambiae* mosquitos [78, 79, 93]. Because the *Plasmodium* chitinase is a potential target for blocking malaria transmission, the idea of developing antibodies to neutralize chitinase activity emanated. A monoclonal antibody (1C3-MAb) was generated against the recombinant *P. falciparum* chitinase PfCHT1. The 1C3-MAb indeed inhibited PfCHT1 activity and significantly reduced the infectivity of the *P. falciparum* parasite in mosquitoes [94, 95]. A recombinant single-chain antibody (scFv) derived from 1C3-MAb hybridoma cells also significantly reduced *P. gallinaceum* parasite transmission to mosquitoes by inhibiting the orthologous PgCHT2 enzyme [96]. The fact that the recombinant 1C3-MAb recognizes PfCHT1 as well as PgCHT2 allows using the avian parasite *P. gallinaceum* as a model system for elucidating the role of chitinases for invasion of the midgut epithelium. More importantly, however, transgenic strains of *Anopheles stephensi* expressing two scFvs directed to the parasite chitinase did not show the development of sporozoites after challenging them with *P. falciparum* [97]. The expression of a dual scFv transgene can completely block parasite development without affecting the mosquito's fitness. Interestingly, antibodies to microfilarial chitinases from *Brugia malayi,* a nematode which causes lymphatic filariasis in humans, also block parasite transmission [98].

4 Recombinant Chitinases in Pest Control

4.1 Chitinases Enhance Virulence of Baculoviral Biopesticides

Chemical pest management strategies may cause contaminations with toxic compounds that have problematic effects on the environment and eventually affect animal and human health. In addition, the extensive use of pesticides generates resistances that render many of the widely used insecticidal compounds ineffective. Therefore, alternate concepts of integrated pest management (IPM) have been

developed, including nonchemical strategies to control insects. A major objective of IPM is applying different methods to produce synergistic effects [99]. Pest populations are not controlled by attempting eradication but rather by reducing the number of pests to levels that do not cause economic damage. The methods that are combined in IPM strategies include environmentally safe and selective chemical pesticides, use of crop cultivars with pest resistance, intelligent cultivation practices, physical methods, natural biopesticides, and biocontrol with natural predators, parasites, and microbial organisms and agents.

Baculoviruses have been recognized as efficient biopesticides for the control of lepidopteran insect species in agriculture and forestry. The majority of baculoviruses used as biopesticides belong to the group of nucleopolyhedroviruses (NPV; α-baculoviruses); some are granuloviruses (GV; β-baculoviruses). These viruses exhibit high species-specificity and lack negative impact on plants, mammals, birds, fish, or even on nontarget insects. This is especially desirable when beneficial insects need to be protected or when an ecologically sensitive area is being treated. However, the narrow host range of baculoviruses and their slow working mode limit their use in pest control to some extent. Baculoviruses encode proteases and chitinases, which specifically degrade the protective PM in the midgut of infected insects; this presumably increases virulence and infectivity and mediates liquefaction of the integument. For instance, the *Mamestra configurata* (bertha armyworm) NPV (McNPV-A) encodes a metalloprotease enhancin, which evidently degrades PM proteins [100]. Chitinases are encoded by many baculoviral genomes, including those of the *Autographa californica* multicapsid NPV (AcMNPV) [101], *Spodoptera litura* multicapsid NPVs (SlMNPVs) [102], *Helicoverpa armigera* singlecapsid NPV (HaSNPV) [103], *Antheraea pernyi* NPV (ApNPV) [104], *Epiphyas postvittana* NPV (EpNPV) [105], *Orgyia pseudotsugata* multicapsid NPV (OpMNPV) [106], *Bombyx mori* NPV (BmNPV) [107] and the *Cryptophlebia leucotreta* granulovirus (ClCV) [108]. The function of the AcMNPV chitinase ChiA has been extensively studied, both in vitro and in vivo. The viral ChiA was expressed in *Sf*9 cells and purified using a pepstatin-aminohexyl Sepharose column [48]. Careful analyses of enzyme activity and cleavage products revealed that the mode of action was similar to that of chitinase A from the enterobacterium *Serratia marcescens* (SmChiA). It hydrolyzed the second β-(1,4) glycosidic bond from the non-reducing end of chitooligosaccharide substrates and acted also on solid β-chitin in a processive mechanism.

The viral chitinase seems to promote liquefaction of the larvae, a process that is usually observed in the late period of a baculoviral infection and involves partial degradation of the integument by proteolytic and chitinolytic activities. The processive working mode of ChiA may be especially advantageous for liquefaction. In AcMNPV-infected larvae of *Trichoplusia ni*, liquefaction depends, next to ChiA, on the baculoviral endoprotease cathepsin (v-Cath) [47], which physically interacts with the viral ChiA before being released from infected cells [109]. Site-directed mutagenesis of the ChiA active site residues D311 and E315 in the recombinant baculovirus caused a reduction in chitinolytic activity and an attenuated liquefaction of host larvae, indicating that ChiA is an important virulence factor of baculoviruses

[110]. Interestingly, ChiA contains a C-terminal KDEL motif retaining the protein in the endoplasmic reticulum [111]. When the nucleotide sequence encoding the KDEL motif was deleted in the viral chitinase gene, and subsequently *T. ni* cells were infected with the recombinant virus, the chitinase localized at the plasma membrane and was secreted into the supernatant of the culture medium. Consistently, the biological activity was enhanced in *T. ni* larvae infected with the recombinant virus encoding a KDEL-deficient chitinase version [112]. Why ChiA is retained in the endoplasmic reticulum to some extent is unknown, but it may attenuate liquefaction until enough polyhedra have been produced to embed the virions. The observed interaction of ChiA with v-Cath may be required for coordinating proteolytic and chitinolytic activities for liquefaction [109].

The potential of the ChiA chitinase as a biopesticide has been further explored by Rao et al. [113]. The *ChiA* gene was expressed in *Escherichia coli* cells and purified by affinity chromatography. When isolated peritrophic matrices from *B. mori* larvae were incubated with increasing amounts of the recombinant chitinase, a dose-dependent increase in PM permeability and number and size of PM perforations was observed. Feeding of the recombinant chitinase to *B. mori* larvae resulted in 100 % mortality at high doses, whereas lesser doses reduced larval growth. This finding implies that optimization of the baculoviral genome to increase chitinase expression by using alternate promoters may provide a powerful means for the development of environmentally safe biopesticides [114]. As insect chitinases have been optimized during evolution to degrade cuticle and peritrophic matrices, the insecticidal activities of baculoviruses may be improved by generating recombinant viruses encoding an insect chitinase with desired properties. One example supporting this hypothesis will be discussed in Sect. 4.3.

4.2 Chitinases Increase Insecticidal Activities of Biopesticides

Among the most successful biopesticides are the δ-endotoxins (Cry toxins) produced by *Bacillus thuringiensis*. After their release from spore crystals in the midgut, they bind to their specific receptors at the apical membrane of midgut epithelial cells and damage the membrane by pore formation, so that the cells eventually lyse [115]. Before the Cry toxins can interact with the apical membranes, they have to pass the chitin-containing PM, which forms a physical barrier. It was suggested that chitinases increase the larvicidal effects by perforating the PM, improving the accessibility of the Cry toxin to the epithelial membrane. An obvious idea was to coapply Cry toxins and chitinases, which indeed increased the insecticidal effect of *B. thuringiensis* on *Choristoneura fumiferana* larvae [116]. Also, the co-application of *B. thuringiensis* spore crystal suspension together with chitinolytic bacteria yielded significant synergistic insecticidal effects against *Spodoptera littoralis* larvae [117]. It was also shown that the addition of *Serratia marcescens* chitinase protein preparations to Cry toxin preparations caused synergistic toxic effects in *Spodoptera littoralis* larvae [118]. More recently,

recombinant plasmids containing the cry1Ac gene from *B. thuringiensis* and chitinase-encoding genes from tobacco *(Nicotiana tabacum*) were generated to transform acrystalliferous *B. thuringiensis*. The transformed bacteria exhibited significant chitinase activity, and when the insecticidal activity of the transformed bacteria was evaluated using *Helicoverpa armigera* larvae, it was more than tenfold higher compared to the bacteria that produced only the Cry toxin [119]. Even fusion proteins composed of a chitinase and Cry1Ac expressed by *B. thuringiensis* strains have been shown to increase slightly toxicity in *Ephestia kuehniella* larvae in comparison to wild-type strains [120].

Similar approaches have been undertaken to increase the insect virulence of entomopathogenic fungi that are used in biocontrol of insect pests. The genome of the entomopathogenic fungus *Metarhizium anisopliae* encodes several chitinases. One of these chitinases, chitinase 2 (Chi2), is involved in the pathogenicity of this fungus. Strains that overexpress Chi2 showed higher efficiency to kill its host, the cotton stainer bug *Dysdercus peruvianus*, whereas strains defective in the gene encoding Chi2 decreased infectivity of the fungus [121]. Likewise, a transgenic *Trichoderma konigii* strain expressing the chitinase 42 from *Metarhizium anisopliae* showed insecticidal activity against the Asian corn borer, *Ostrinia furnacalis* and the silkworm *B. mori* [122]. More recently, Araújo et al. showed that feeding of a bacterial chitinase disrupts the PM and reduces fecundity of female sandflies (*Lutzomyia longipalpis*), which are important vectors of visceral leishmaniasis [123]. Again, it is tempting to speculate that the use of group IV insect chitinases that are expressed in the midgut might increase the synergistic effects of Cry toxins or the insect virulence of entomopathogenic fungi.

4.3 Insect Chitinases as Biopesticides

To date, only a few studies have been reported that directly used insect-derived chitinases as biopesticides for the control of pests. Gopalakrishnan et al. have constructed a recombinant AcMNPV baculovirus expressing a group I chitinase from *M. sexta* under the control of the polyhedrin promoter [124]. When the fourth instar larvae of *M. sexta* or *Spodoptera frugiperda* were injected with the recombinant virus, the chitinase was detectable in large amounts in the hemolymph. Liquefaction of infected *S. frugiperda* larvae occurred significantly earlier than when the insects were infected with a wild-type virus, indicating increased insecticidal activity. Similarly, a recombinant AcMNPV expressing a group III chitinase from the hard tick *Haemaphysalis longicornis* showed bioacaricidal effects against ticks when topically applied [125]. A mixture of recombinant virus and the purified recombinant protein was found to be more efficient in killing the ticks than the recombinant virus and pure chitinase alone. Mice that were immunized with the recombinant purified chitinase from *H. longicornis* developed a specific protective anti-tick immune response affecting tick molting [126]. These findings suggest that recombinant chitinases may be efficient antigens for

vaccination to biologically control ticks. Similar strategies may be helpful also to develop chitinase-based vaccines that block the transmission of leishmaniasis by sandflies [127, 128]. Interestingly, even the purified recombinant chitinase has insecticidal effects. Fitches et al. produced a recombinant chitinase from the tomato moth, *Lacanobia olerace* in the yeast, *Pichia pastoris*, and injected it into *L. oleracea* larvae [129]. They observed 100 % mortality already at a comparable low dose and a reduction in cuticle thickness when injected prior to molting. When fed orally, larval growth and food consumption were reduced.

Insect-derived chitinases may become also important for bioprocessing industries that, for instance, produce chitooligosaccharides with defined chemical properties. They have been expressed in different heterologous systems and purified in reasonable amounts. However, biotechnological processes will require cost-efficient large-scale production of highly active preparations. Most of the studies expressing insect chitinases so far used *E. coli* strains [102, 130–132] or insect cells [38, 39, 133]. Because insect chitinases may likely exhibit eukaryotic posttranslational modifications that improve folding and activity, cost-efficient eukaryotic cells such as the yeast *Pichia pastoris* could be the right expression system of choice to produce a high yield of stable and highly active preparations of genetically optimized enzymes for bioprocessing applications.

5 Chitinase Transgenes in Crop Protection

5.1 Transgenic Plants Expressing Chitinases

Because of the nematocidal, fungicidal and insecticidal properties of chitinases, transgenic plants were generated that heterologously express GH18 and GH19 chitinases from various baculoviral, bacterial, and insect sources to increase plant resistance [25, 134–137]. The high potential for transgenic plants expressing chitinases for use in plant protection against herbivorous insects has been established. The first study that evaluated insect resistance of transgenic plants expressing an insect chitinase was published in 1998. In this study, a transgenic tobacco was generated producing a group I chitinase from *M. sexta* [138, 139]. Although expression levels appeared to be generally low and the recombinant chitinase was truncated, larvae of the tobacco budworm *Heliothis virescens* were impaired in their growth when feeding on the leaves of the transgenic tobacco. Moreover, the larvae showed a higher mortality and caused less feeding damage compared to larvae fed on control plants. However, no effects on growth and mortality were observed for *M. sexta* larvae. This result may be explained by the greater thickness of the PM in the case of *M. sexta* larvae, indicating that susceptibilities to toxic chitinases may vary among different insect species. However, when the leaves of transgenic tobacco were sprayed with a formula containing a sublethal dose of Cry toxins from *B. thuringiensis*, a synergistic insecticidal effect

was observed in *H. virescence* larvae, as well as *M. sexta* larvae. The *M. sexta* chitinase was also expressed in transgenic cotton, which reportedly conferred insect resistance in the field. The chitinase was detectable in leaf tissues using antibodies developed to the recombinant enzyme [132].

To increase toxic effects on feeding insects, the gene encoding a scorpion insect toxin from *Buthus martensii* (BmkIT) was expressed in combination with the *M. sexta* chitinase in transgenic *Brassica napus* [140]. Some of the obtained transgenic plants showed high expression levels for both chitinase and scorpion toxin, and conferred significant resistance to the diamondback moth, *Plutella maculipenis*. Synergistic effects for the chitinase and the scorpion insect toxin have not been tested in this study; hence, it is not clear to what extent the chitinase contributes to the observed mortality. In an attempt to improve the resistance of papaya plants to the carmine spider mite (*Tetranychus cinnabarius*), McCarthy et al. generated a transgenic line of *Caprica papaya* expressing the *M. sexta* chitinase [141]. The transgenic papaya line showed increased tolerance to the spider mites under field conditions.

Likewise, chitinases from baculoviral sources have been used to generate transgenic plants. Although Shi et al. observed no insecticidal effects when *H. virescens* larvae were fed on transgenic tobacco leaf tissue expressing the baculoviral chitinase ChiA from AcMNPV, they reported significant tolerance against the fungal pathogen *Alternaria alternata* [142]. In contrast, Corrado et al. showed that transgenic tobacco plants expressing an active ChiA protein from AcMNPV are significantly protected against fungal pathogens (*Botrytis cinerea* and *Alternaria alternate*) and lepidopteran larvae (*B. mori*). However, they observed no insecticidal effects on aphid *M. persicae* populations [143]. As indicated by the latter two examples, the use of transgenic plants expressing chitinases has to be discussed carefully, particularly also because probiotic effects on insect pests have been reported in transgenic plants expressing chitinases. For instance, transgenic potatoes expressing a chitinase from the coleopteran pest *Phaedon cochleariae* revealed slightly positive effects on population growth of the aphid *M. persicae* [144]. Because aphids do not form a PM, they may be considered as nontarget insects for transgenic plants expressing baculoviral or insect chitinases.

Although some of the results obtained with transgenic plants expressing insect chitinases are inconsistent, it seems plausible that chitinase-mediated resistance can be improved by considering some of the recent insights on the different biochemical properties and physiological functions of chitinases. For example, so far only group I insect chitinases have been used for the construction of transgenic plants. However, these enzymes are located in the integument, where they have important function during molting. The primary target of plant-expressed chitinases, however, may be considered the PM in the midgut (in contrast to the chitinases acting systemically during a baculoviral infection). Therefore, it seems promising to use group IV chitinases as transgenes, which evidently are expressed

in the insect gut and hence may have higher chitinolytic activities due to evolutionary adaptations to this proteolytic environment. These adaptations may include resistance to proteolysis, distinctive pH optima, and different working modes that allow degradation of chitinous substrates which differ in the type of chitin and associated proteins.

Finally, it has to be noted that some of the observed insecticidal effects may be mediated by the stimulation of general defense mechanisms triggered by the exogenous chitinase rather than by direct chitinolytic action of the transgene. In addition, legal restrictions and problems with public acceptance of transgenic plants in some countries may raise concerns.

5.2 Insect Chitinases as Target Genes for RNAi

Using RNA interference as a powerful tool to investigate gene functions, a few studies revealed vital functions for chitinases for growth and development of insects. Zhu et al. reported lethal effects for the injection of the group I chitinase TcCHT5, the group II chitinase TcCHT10, and the group V IDGF protein TcIDGF4, whereas the injection of dsRNA for the group III chitinase TcCHT7 resulted in severe wing and elytral abnormalities [55]. Similar results were obtained when dsRNA specific for a group I and a group IV chitinase from the beet armyworm *Spodoptera exigua* was injected into pupae of this lepidopteran pest [145]. The injection of dsRNA specific for either chitinase led to a significant reduction in survival rates. Although the injection of dsRNA specific for the group I chitinase led to molting defects, that for the group IV chitinase prevented adult eclosion. Using a feeding-based RNAi approach, Khajuria et al. were successful in reducing transcript levels for a group IV chitinase in the European corn borer, *Ostrinia nubilalis*, which is predominantly detected in midgut of wild-type larvae [146]. In response to RNAi-mediated knock-down of transcript levels, the chitin content in the PM was slightly increased and larval growth was significantly impaired compared with control larvae. These findings indicate that the examined group IV chitinase is required for proper PM formation. If this function is disturbed, growth and development of the *O. nubilalis* larvae are negatively affected. As documented by the latter example, the knockdown of transcripts was accomplished by feeding dsRNA to larval pests. This approach raises the possibility of directly spraying stabilized dsRNA formulation on host plants to silence the expression of vital genes in herbivorous insect pests. However, the stability of the dsRNA as well as the cost efficiency in synthesizing such dsRNA-based insecticides may be problematic. The expression of corresponding dsRNAs in transgenic plants, however, may be more promising, as this approach has been shown to provide protection against various pests [147].

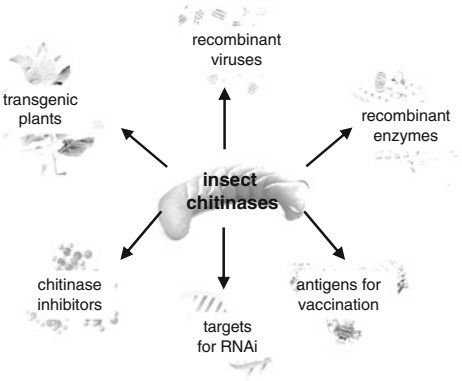

Fig. 4 Potential use of insect chitinases as biopesticides and targets for RNA interference, inhibitor- and antibody-based strategies of pest control

6 Conclusions

During the past decades, knowledge about the structure and function of insect GH18 chitinases has significantly increased. Next to the insights gained into their phylogenetic relationships, regulation, and working modes, significant progress has been made in understanding their distinctive physiological roles within different insect tissues. It became evident that chitinases are involved in a variety of physiological processes including molting, digestion, cell proliferation, and immune responses. Because of the inhibitory effects on the growth and development of fungi, parasites, and insects, chitinases from predominantly noninsect sources have been established as biopesticides and transgenes in crop protection, and as antigens for vaccination programs in animal and human health. Insect chitinases, however, may be considered as a largely unexploited resource for various applications in insect biotechnology due to their biochemical diversity resulting from evolutionary adaptations (Fig. 4). Improved understanding of their structure and biochemistry will accelerate their usage in biotechnological processes. Applications will include also recombinant insect chitinases that are expressed in heterologous systems and genetically optimized for bioprocessing industries.

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Cellulases from Insects

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Abstract Bioethanol is currently produced by the fermentation of sugary and starchy crops, but waste plant biomass is a more abundant source because sugars can be derived directly from cellulose. One of the limiting steps in the biomass-to-ethanol process is the degradation of cellulose to fermentable sugars (saccharification). This currently relies on the use of bacterial and/or fungal cellulases, which tend to have low activity under biorefinery conditions and are easily inhibited. Some insect species feed on plant biomass and can efficiently degrade cellulose to produce glucose as an energy source. Although insects were initially thought to require symbiotic relationships with bacteria and fungi to break down cellulose, several species in the orders Dictyoptera, Orthoptera, and Coleoptera have now been shown to produce their own cellulases in the midgut or salivary glands, and putative cellulase genes have been identified in other orders. Insect cellulases often work in concert with cellulases provided by symbiotic microbiota in the gut to achieve efficient cellulolysis. We discuss the current status of insect cellulases and potential strategies that could be used to find novel enzymes and improve their efficiency.

Keywords Biofuels · Cellulase · Cellulose · Ethanol · Insects · Saccharification

Abbreviations

CMC	carboxymethylcellulose
GH	glycoside hydrolase

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1 Ethanol from Cellulose

Ethanol is the most widely used biofuel in the world. It is produced mainly using first-generation processes; that is, by the fermentation of sugar and starch derived directly from crops such as wheat, corn, sugar beet, sugar cane, molasses and fruits/vegetables. This generates only a small positive energy yield when the energy input costs are balanced against the energy value of the resulting fuel [1, 2]. However, ethanol can also be produced indirectly from these and other crops by using the waste biomass (e.g., corn stover) if the lignocellulose can also be converted into sugars [3]. Such second-generation biofuels have a large positive energy yield when energy inputs and the energy value of the resulting fuel are balanced. For example, second-generation ethanol from corn stover has 10 times the fossil energy ratio of ethanol derived from corn starch [1, 2].

Cellulosic ethanol can be produced from almost any plant biomass, but favorable sources include wood chips, corn stover, grasses such as switchgrass (*Panicum virgatum*) and *Miscanthus* spp., garden clippings, and food waste. Lignocellulose as a raw material is thus abundant, widely distributed, often free, and readily replenished and sustainable because plants produce lignocellulose by first converting CO_2 into sugars and then into polymers using the energy from sunlight. However, the production of ethanol from cellulose requires a greater amount of processing than first-generation biofuels fermented directly from starch and sugars. Specifically, the bottlenecks are the reduction of biomass to particles small enough to make enzymatic conversion efficient (i.e., comminution) followed by the conversion of lignocellulose into sugars ready for fermentation (i.e., saccharification) [4]. The tight packing of cellulose fibers makes this an energy-intensive process, although it can be achieved by a combination of mechanical and chemical processing, followed by enzymatic processing using cellulases [5].

Pretreated lignocellulosic materials can be enzymatically hydrolyzed under relatively mild conditions (e.g. 50 °C and pH 5) without the formation of inhibitory byproducts. All major pretreatment methods require an enzymatic hydrolysis step to yield enough sugars for cost-efficient fermentation, and although small-scale operations remain the state of the art, several companies are now exploring the means to scale up from the laboratory to pilot-scale comminution and saccharification processes [6–8].

2 Cellulases

2.1 Classification and Activity

Cellulose is an abundant polymer comprising β(1,4)linked D-glucopyranosyl monomers. Enzymes that degrade cellulose by hydrolyzing the β-1,4 linkage joining two glucose molecules are known as cellulases, but there are several distinct types with different activities. Organisms that can use cellulose often produce multiple enzymes with different substrate specificities or cooperate with other organisms producing their own cellulases to break down complex lignocellulosic products such as wood [6].

Cellulases have nonlinear kinetics on polymeric substrates but show Michaelis–Menten kinetics on small soluble substrates [9]. Most cellulases are endocellullases (EC 3.2.1.4), which have an open active site so they can bind and cleave a cellulose molecule at any accessible point along the chain [10]. Endocellulases bind randomly along a cellulose molecule, make a few cleavages and then dissociate from the chain. In contrast, exocellulases (EC 3.2.1.91) are characterized by an active site inside a tunnel and they bind only at one end of a cellulose chain. They remain bound to a cellulose chain, processively cleaving cellobiose residues until they dissociate [11]. There are two types of exocellulases—one that attacks the reducing end of cellulose molecules and one that attacks the nonreducing end [12]. A third class of cellulases, currently known only in bacteria, carries out an initial endocellulytic attack but then processively attacks the nonreducing end to release cellotetraose. Finally, cellobiases/β-glucosidases (EC 3.2.1.21) hydrolyze the fragments generated by exocellulases and generate individual monosaccharides.

There are at least eleven distinct cellulase families based on sequence and structural homology, and eight different folds are recognized, mirroring the structural diversity of the substrate [13]. Many but not all cellulases have a carbohydrate-binding domain in addition to the catalytic domain, depending on the source organism and its cellulose attack strategy (see below).

2.2 Microbial Sources of Cellulase

Although most enzymes have soluble substrates that diffuse into their active sites, the situation is reversed for cellulases, which must diffuse to their insoluble substrate and pull a polymer chain into the active site to achieve cleavage. Many aerobic microorganisms use the free cellulase mechanism involving the secretion of a set of individual cellulases, which then act synergistically to degrade cellulose [14]. Cellulase synergism can increase the specific activity of appropriate mixtures by up to 15-fold compared to individual cellulases [15].

Many anaerobic microorganisms instead use large multienzyme complexes known as cellulosomes to degrade cellulose [16]. Only a few of the enzymes in

cellulosomes contain a carbohydrate-binding domain, which is instead provided by the scaffold of the cellulosome, helping to bind the entire complex to the substrate. However, a small number of anaerobic cellulolytic thermophillic bacteria, such as *Caldicellulosiruptor* spp., secrete free cellulases with carbohydrate-binding domains and can hydrolyze plant biomass without pretreatment, unlike most other cellulolytic microorganisms [17].

At least two cellulolytic bacteria (the rumen anaerobe *Fibrobacter succinogenes* and the soil aerobe *Cytophaga hutchinsonii*) use neither the free cellulase nor the cellulosome strategy, and the precise cellulolytic mechanism remains unclear [18]. A number of recently discovered fungal genes appear to encode enzymes with weak cellulase activity that stimulate the activity of other cellulases under certain conditions or help to loosen the cellulase substrate to facilitate penetration by conventional cellulases [19].

2.3 Insect Sources of Cellulase

Many xylophagous (wood-eating) insects have symbiotic protozoa and/or bacteria in their digestive system, which assist in the breakdown of cellulose; others are vectors for fungi that grow on wood, allowing the insects to seed their food source with cellulolytic microbes [20]. The first endogenous insect cellulase was discovered in 1998 in the termite *Reticulitermes speratus*, which was shown to retain its ability to feed on wood even when its gut fauna were destroyed [21]. The traditional view that animals cannot produce their own cellulase and must rely on gut microorganisms to hydrolyze cellulose has thus been overturned.

The *Reticulitermes speratus* cellulase (RsEG) encodes an endo-β(1,4)-glucanase [21]. Since this discovery, examples of endogenous cellulases have been reported in three different orders of insects: Blattaria (cockroaches and termites) [22–26], Coleoptera (beetles) [27–31], and Orthoptera (crickets) [32]. Insect genome sequencing data have provided additional evidence for putative cellulase genes in Phthiraptera (lice), Hemiptera (aphids), and Hymenoptera (honeybees and wasps) [33]. Thus far, cellulase genes or their homologs have been found in 31 species spanning six orders of insects, and the list is growing rapidly (Table 1).

3 Evolution of Insect Cellulase Genes

Cellulase genes encoding class 9 glycosyl hydrolases (GH9) are distributed in all the insect orders examined thus far (Table 1). The corresponding enzymes differ extensively in structure and sequence from microbial cellulases; it is therefore considered unlikely that they reflect the consequences of horizontal gene transfer. Indeed, it has been proposed that a common ancestor of animals possessed a GH9 cellulase gene because they are widespread among the invertebrates, appear to be

Table 1 List of insect species with putative cellulase genes (updated from [20])

Order and species	Common name	Family	Cellulase type	Cellulase class
Orthoptera				
Teleogryllus emma	Emma field cricket	Gryllidae	Endo-β-1,4-glucanase	GH9
Blattaria				
Polyphaga aegyptiaca	Egyptian desert cockroach	Polyphagidae	Endo-β-1,4-glucanase	GH9
Blattella germanica	German cock-roach	Blattellidae		
Panesthia angustipennis spadica	(none)	Blaberidae		
Panesthia cribrata	Australian cockroach	Blaberidae		
Salganea esakii	(none)	Blaberidae		
Periplaneta americana	American cockroach	Blattidae		
Cryptocercus clevelandi	Cockroach	Cryptocercidae		
Mastotermes darwiniensis	Giant northern termite	Mastotermitidae		
Hodotermopsis sjoestedti	Damp wood termite	Termopsidae		
Neotermes koshunensis	(none)	Kalotermditae		
Reticulitermes speratus	Japanese termite	Rhinotermitidae		
Reticulitermes flavipes	Eastern subterranean termite	Rhinotermitidae		
Coptotermes formosanus	Formosan subterranean termite	Rhinotermitidae		
Coptotermes acinaciformis	Australian subterranean termite	Rhinotermitidae		
Odontotermes formosanus	Black-winged subterranean termite	Termitidae		
Nasutitermes takasagoensis	(none)	Termitidae		
Nasutitermes walkeri	(none)	Termitidae		
Sinocapritermes mushae	(none)	Termitidae		
Phthiraptera				
Pediculus humanus humanus	Body louse	Pediculidae	Endo-β-1,4-glucanase	GH9
Hemiptera				
Acyrthosiphon pisum	Pea aphid	Aphididae	Endo-β-1,4-glucanase	GH9
Lygus lineolaris	Tarnished plant bug	Miridae		GH9

(continued)

Table 1 (continued)

Order and species	Common name	Family	Cellulase type	Cellulase class
Coleoptera				
Tribolium castaneum	Red flour beetle	Tenebrionidae	Endo-β-1,4-glucanase	GH9
Apriona germari	Mulberry longhorn beetle	Cerambycidae		GH5 and GH45
Psacothea hilaris	Yellow-spotted longicorn beetle	Cerambycidae		GH5
Oncideres albomarginata chamela	Borer beetle	Cerambycidae		GH5 and GH45
Anoplophora malasiaca	White spotted longhorn beetle	Cerambycidae		GH5
Phaedon cochleariae	Mustard beetle	Chrysomelidae	Endo-β-1,4-xylanase	GH45
Hypothenemus hampei	Coffee berry borer	Curculionidae		
Hymenoptera				
Apis mellifera	Honey bee	Apoidae	Endo-β-1,4-glucanase	GH9
Nasonia vitripennis	Jewel wasp	Pteromalidae		GH9

monophyletic [34], and share similar intron/exon boundaries [35, 36]. It is therefore also conceivable that a common ancestor of insects possessed a GH9 cellulase gene and that the GH9 gene was vertically transferred to the extant insects.

In contrast, GH5 and GH45 class cellulases have only been found in the Coleoptera [27–31] (Table 1). GH5 and GH45 cellulases have the same substrate specificity as GH9 cellulases but the structures are distinct, suggesting the different classes have arisen by convergent evolution. It is not clear whether the GH5 and GH45 genes were transferred vertically from the common ancestor of insects to the coleopterans or horizontally from more distantly related organisms (GH5 and GH45 cellulase genes are also present in close relatives of arthropods, such as nematodes and mollusks). Further data from sequencing projects will no doubt shed light on the phylogenetic relationships among these sequences.

Cellulase genes are unlikely to be stable in genomes because they have been lost from the genomes of model insects such as *Anopheles gambiae*, *Drosophila melanogaster,* and *Bombyx mori* [33, 37], and probably from many other species [35]. Cellulase genes are also absent from the recently-sequenced genomes of two butterflies, the monarch *Danaus plexippus* and the postman *Heliconius melpomene* [38, 39]. A putative GH9 cellulase gene is present in the genome of the red flour beetle *Tribolium castaneum* [40]. The unstable nature of cellulase genes is likely to frustrate research in this field, and many more insect genomes will need to be sequenced to derive firm conclusions about the evolutionary origins of insect cellulase genes.

A small number of β-glucosidase sequences have also been identified in insects, including noncellulolytic species such as *B. mori* [41] (these are not included in Table 1). The sequences are homologous to GH1 except for a GH3 homolog from the midgut of *Hodotermopsis sjostedti* revealed by transcriptome analysis [42]. The universal occurrence and multiple functions of β-glucosidases in animals suggests that the corresponding genes have probably been conserved during the evolution of insects.

4 Screening for Insect Cellulase Genes

Two major strategies have been used to isolate insect cellulase genes, namely sequence mining and functional assays. Sequence mining is a bioinformatics approach that involves the screening of genomic, transcriptomic, or proteomic data for cellulase signatures, based on sequence and structural homology. With the advent of ultra-high-throughput methods such as next-generation DNA sequencing, transcriptomic analysis based on RNA-seq methods, and proteomics based on the rapid *de novo* sequencing of peptides, the sequence-mining approach provides rapid results from any species of interest. Genomic analysis is advantageous because it can reveal cellulase genes that are not expressed (as well as evolutionary artifacts such as pseudogenes), whereas transcriptomics and proteomics are

advantageous because they allow targeted screening based on conditional expression (e.g., genes induced by feeding on wood). Many putative insect cellulases have been identified in this manner, but it is necessary to ensure that contaminating microbial nucleic acids and proteins do not generate false-positive results and that insect genes and microbial genes can be distinguished (e.g., on the basis of codon bias or promoter structure) [33].

Functional assays may involve the direct screening of gut extracts (preferably a comparison between guts that have been freshly isolated and those purged of natural flora) or the expression-based screening of genes identified by sequence mining. For functional analysis, the choice of substrate is important. Carboxymethylcellulose (CMC) is a soluble form of cellulose that is an excellent substrate for endocellulases and its hydrolysis does not require a carbohydrate-binding domain. Amorphous cellulose, which is produced by concentrated acid treatment of crystalline cellulose, is a good substrate for most cellulases and its hydrolysis is usually not affected by the carbohydrate-binding domain. Crystalline cellulose (Avicel, bacterial cellulose, or filter paper), which is the main form of cellulose in most plant cell walls, requires a carbohydrate-binding domain for effective hydrolysis [43].

5 Strategies for the Improvement of Insect Cellulases

Once cellulase genes have been isolated, several strategies are available to improve their activity using gene shuffling and/or random or directed mutagenesis as a basis for protein engineering. For example, a mutant termite cellulase gene has been expressed in bacteria by shuffling between four orthologous cDNAs [44]. Although many mutagenesis techniques that allow the efficient generation of diversity have been developed, the major bottleneck has been the selection of improved variants because most high-throughput assays select for binding interactions rather than enzyme activity [45–47].

Several cellulase activity assays are available using natural or synthetic substrates as described above, but few of these are suitable for high-throughput screening. Ideally, such assays would generate fluorescent products suitable for fluorescence-activated cell sorting (FACS), which has an ultra-high-throughput capacity of $>10^7$ clones per hour compared to 10^3–10^5 clones per hour for microtiter plate assays and 10^4–10^6 clones per hour for solid-phase assays [48–51]. Several cellulase assays generate fluorescent products; for example, one detects (1,4)-β-glucanase activity using 4-methylumbelliferyl-3-cellobiose, whereas another is based on the detection of released glucose with Amplex Red by means of a coupled glucose oxidase reaction [52, 53]. However, neither are compatible with FACS because the reaction product is not charged and would diffuse from the emulsion compartments.

To address these challenges, a novel ultra-high-throughput screening assay for cellulase activity has been developed based on FACS and double emulsion technology. In this approach, cellulase activity is detected using a series of coupled

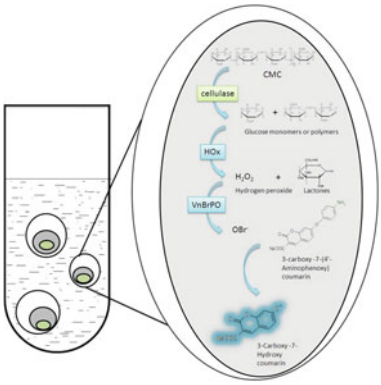

Fig. 1 Coupled cellulose assay, which allows the detection of reducing sugars released by any natural or artificial cellulose substrate

enzymes including hexose oxidase, which generates hydrogen peroxide from the reducing sugars released by cellulases in the presence of any natural or artificial substrate (Fig. 1). In the first description of this assay, a 12-fold enrichment of positive (cellulose-expressing) cells in cellulase reference libraries was achieved after a single sorting round [54].

6 Potential Industrial Applications of Insect Cellulases

Parallels can be drawn between wood-feeding insects and industrial platforms for the production of cellulosic ethanol. Essentially, wood-chewing insects are small-scale processing factories, taking in lignocellulose and producing sugars, albeit then using those sugars for growth and survival rather than for the production of biofuels. Each compartment of the insect gut can be matched to a corresponding industrial process stage, and the efficiency is striking: up to 99 % for cellulose and up to 87 % for hemicellulose [55].

The efficiency of digestion is enhanced by pretreatment that converts biomass into microsize particles. Termite mandibles achieve this by chewing wood into particles smaller than 50 μm in diameter [56, 57]; however, in an industrial process, this must be replaced by mechanical comminution based on progressive chipping, grinding, and/or milling. Thus far, the smallest particles that can be produced mechanically are 0.2–2 mm in diameter. This step is necessary to reduce cellulose crystallinity and make the substrate more accessible to cellulases, but it requires the input of energy that exceeds the energy available in the biomass [58]. In addition to comminution, other energy-consuming pretreatments such as pyrolysis, steam explosion, and ammonia fiber explosion may be needed to weaken and remove lignin and the hemicellulose matrix covering cellulose fibers [58]. There are no counterparts to these steps in termites because the particle size is smaller, and this is sufficient to achieve the necessary weakening.

Current biomass degradation pipelines do not use purified cellulase, which would be too expensive. Instead, they use crude extracts of cellulolytic fungi, such as *Trichoderma reesei* (the main source of commercial-grade cellulase), *T. viride,* and *Aspergillus niger* [59]. These extracts contain multiple cellulases with different specificities, allowing synergic activity between the dominant cellobiohydrolases and the less abundant endo-β(1,4)glucanases to ensure the efficient digestion of insoluble cellulose [60, 61]. In termites, cellobiohydrolases, multidomain cellulases with carbohydrate-binding domains, hemicellulases, and lignin-degrading enzymes are not involved in the initial digestion of cellulose in the midgut, possibly because the mastication process produces such fine particles that such enzymes are unnecessary, or alternatively because symbiotic microbial cellobiohydrolases may fulfill the necessary functions [62].

Another bottleneck in the industrial conversion of cellulose into sugars is the need for cellulases to act at a liquid–solid interface due to the solid nature of the substrate. This limits the effective concentration of cellulase (regardless of the absolute concentration in solution) and makes it dependent on the accessible surface area of the substrate [63]. The number of enzyme molecules acting on the liquid–solid interface is directly affected by the enzyme concentration in the liquid phase, so the volume of the liquid phase should be reduced to ensure that the surface of the substrate is saturated. The small volume of the gut in wood-feeding insects (estimated to be 10–20 nl [64]) combined with the large substrate surface area (due to the small particle size following mastication [56, 57]) goes a long way to explain the extraordinary efficiency of cellulose digestion by insect cellulases. Indeed, one estimate of cellulase activity in the wood-feeding species *C. formosanus* is 10^3–10^4 μmol of reducing sugar min^{-1} ml^{-1} using CMC as the substrate [65].

Comprehensive transcriptome analysis in termites and their symbiotic microbes has led to the emergence of a concept described as the digestome [66], which can be regarded as a metagenomic principle, albeit restricted to the insect digestive system [62]. However, the metagenomic data remain theoretical and such studies now need to be backed up by the isolation of the corresponding proteins, their structural and functional analysis, their localization in the insect digestive system, and their linkage into cooperating cellulase complexes to determine how the highly efficient process of cellulose digestion is regulated in woodfeeding insects. This can be achieved by harnessing the power of a new generation of ultra-high-throughput FACS-based assays for cellulase activity [54], which can also be used to identify more efficient variants generated by molecular evolution. Such improved enzymes must then be combined with optimized industrial processes to fully realize the benefits of second-generation biofuels.

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Optimization of Insect Cell Based Protein Production Processes - Online Monitoring, Expression Systems, Scale Up

Damir Druzinec, Denise Salzig, Alexander Brix, Matthias Kraume, Andreas Vilcinskas, Christian Kollewe and Peter Czermak

Abstract Due to the increasing use of insect cell based expression systems in research and industrial recombinant protein production, the development of efficient and reproducible production processes remains a challenging task. In this context, the application of online monitoring techniques is intended to ensure high and reproducible product qualities already during the early phases of process development. In the following chapter, the most common transient and stable insect cell based expression systems are briefly introduced. Novel applications of insect cell based expression systems for the production of insect derived antimicrobial peptides/proteins (AMPs) are discussed using the example of *G. mellonella* derived gloverin. Suitable in situ sensor techniques for insect cell culture monitoring in disposable and common bioreactor systems are outlined with respect to optical and capacitive sensor concepts. Since scale up of production processes is one of the most critical steps in process development, a conclusive overview is given about scale up aspects for industrial insect cell culture processes.

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Contents

1 Introduction

Over the last decades, insect cells were more and more established for recombinant protein production. Insect expression systems were thereby used for both, basic research and large-scale commercial applications. One key factor for their popularity is their ability to produce large quantities of posttranslational modified eukaryotic proteins in a relatively short time period. A relatively new field of application is the production of insect derived antimicrobial peptides/proteins (AMPs), which are intended as novel therapeutics in medical applications. Further, the handling of insect cells is quite easy compared to mammalian cells which also produce functional posttranslational modified recombinant proteins. This means that insect cells grow in suspension at 28 °C, are tolerant to osmolality and no carbon dioxide is needed for their cultivation [1]. The simplicity of insect cell cultivation makes them very attractive for recombinant protein production.

To exploit the maximum potential of insect cell cultivation, the production process has to be well controlled and monitored. With regard to PAT for pharmaceutical processes it became more and more important to ensure product quality by design and during the entire process. In this context, many online tools have been investigated. Some of them are still routinely used in industrial processes. Beyond the standard parameters temperature, pH and oxygen, the key parameters which have to be monitored are the viable insect cell biomass, the metabolic activity and product quantity.

Scale up of well-established processes to industrial scales is the final challenge for the production of recombinant proteins in insect cells. Therefore the specific cultivation parameters should be kept constant. Geometric similarity of the cultivation vessels in small and large scale eases the scale up procedure. Nowadays simulation tools as CFD help a lot in the understanding and scale up of processes even at different cultivation setups in small and large scales. Scale up of insect cell production is relatively rewarding since insect cells usually grow in suspension. The main point in scale up is to guarantee homogeneity to ensure sufficient access to nutrients.

In the following chapter the main insect cell expression systems are introduced. Issues related to the application of insect cell expression systems for the production of *G. mellonella* derived gloverin, as an example for insect derived AMPs, are discussed. Modern online monitoring tools are presented and valued for their application in insect cell processes. Finally, scale up considerations for BEVS based production systems are reflected.

2 Recombinant Protein Production in Insect Cells

The possibilities to produce recombinant proteins in insect cells are manifold. A multiplicity of expression systems, cell lines and vectors are available. Nevertheless the majority in industrial production uses the baculovirus expression system with great success. Recently stable expression systems became prominent for insect cells as well. Compared to mammalian cells or bacteria, the insect cells combine the advantages of relatively short production times and the ability to produce complex post-translational modified proteins.

2.1 Common Insect Cell Lines for Recombinant Protein Production

Insect cell culture began with the establishment of an insect cell line from the pupal ovarian tissue of the moth *Antheraea eucalypti* in 1962 [2]. In the meanwhile over 500 insect cell lines from several species and tissue sources have been established. These cells are widely used in research. However, the number of cell lines used and engineered for recombinant protein production in routine industrial

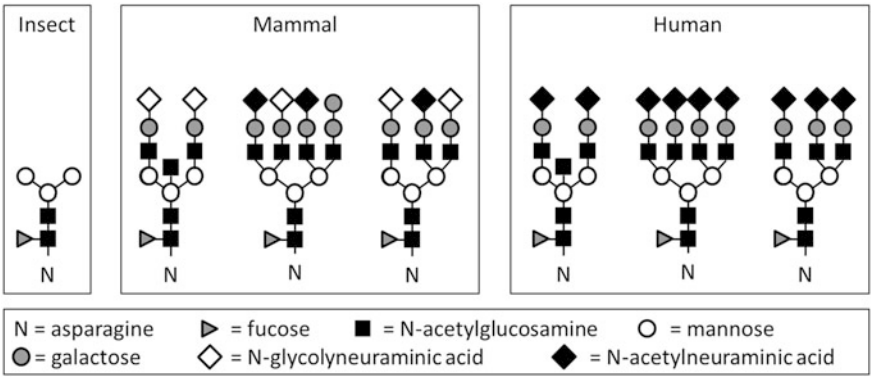

Fig. 1 Major differences between human, mammalian and insect glycosylation pattern

processes is relatively low [3]. In the following, only the most common insect cell lines utilized for recombinant protein production are introduced.

2.1.1 *Spodoptera frugiperda* Derived Cells

Sf9 and Sf21 cell lines are traditional cell lines originally isolated from the pupal ovarian tissue of the fall armyworm, *Spodoptera frugiperda* [4, 5]. The Sf9 cell line is thereby a clonal isolate of IPLBSF21-AE (Sf21 cells) [4]. Both cell lines are spherical with some granular appearance, whereas Sf9 cells are more regular in size. Reported doubling times for Sf21 and Sf9 cells vary between 26–30 h and 24–31 h, respectively [6]. Both cells grow well in monolayer and suspension and are adaptable to serum- and protein-free medium. They are both suitable for viral transfection, resulting in high virus stocks and expression of recombinant proteins. In some cases Sf21 cells may express more protein than Sf9 cells [7].

Functionality of proteins is often dependent on the right glycosylation pattern. Glycosylation in insect cells is different and less complex compared to mammalian cells. In general N-glycans of insect cell proteins mainly contain high mannose or trimannose structures which are truncated and unsialylated (Fig. 1). This pauci-mannose-type is very ordinary for invertebrates. In contrast O-linked glycosylation of insects is similar to mammalian cells [8].

To guarantee full functionality of recombinant insect-produced proteins, a special cell line was developed (SfSWT-1). These cells are transgenic Sf9 cells that have been engineered to produce recombinant proteins with terminally sialylated N-glycans like those found in mammalian systems [9]. Their doubling time and growth characteristics are identical to Sf9 cells except for the fact that serum is required for cell cultivation.

2.1.2 *Drosophila melanogaster* Derived Cells

The most common used *Drosophila melanogaster* cell line is the Schneider 2 cell line, usually abbreviated as S2 cells. These cells have been derived from a primary culture of late stage (20–24 h) *D. melanogaster* embryos [10]. The cell phenotype has many characteristics of cells from a macrophage-like linage. The cells are spherical in shape and possess a granular cytoplasm. Similar to the *S. frugiperda* derived cell lines, S2 cells can grow as loose, semi-adherent monolayer in tissue flasks or suspension culture in dynamic systems, with doubling times around 24 h. Optimum temperature is 28 °C and CO_2 is not required for pH regulation during cultivation. Cultivation in serum-free and even protein-free medium is possible for these cells. Beside S2 cells, other *D. melanogaster* cell lines such as S3 cells and a few derivatives of the Kc cell line were used for recombinant protein production [11].

2.1.3 Other Cells

The High Five or Tn5B1-4 cell line has been established from the ovarian cells of the cabbage looper, *Trichoplusia ni* [12]. A characteristic of this cell line is a population doubling time of less than 24 h. The cells grow in adherent culture, but form irregular monolayers. Suspension culture and serum-free cultivation is possible. Compared to Sf9 cells, High five cells provide 5–10 fold higher secreted recombinant protein expression [13]. However, this high productivity may be more evident in low passage cells [12].

2.2 Expression Systems for Insect Cells

Insect cells can express recombinant proteins via transient and stable expression. In the case of insect cells, transient expression mostly means lytic expression where cells were infected with a lytic recombinant virus carrying the gene of interest. Stable expression in insect cells is similar to the stable expression in mammalian cells, with an insertion of the expression vector followed by a selection and screening of high producer clones. The following section focuses on the introduction of the most prominent insect cell expression systems.

2.2.1 Baculovirus Expression Vector System (BEVS)

Many types of viruses can infect insect cells. The most common ones belong to the family of *Baculoviridae*. The BEVS used today are based on the *Autographa californica* multiple nucleopolyhedrovirus (*AcM*NPV) which mainly infects *Lepidoptera* derived insect cells. Therefore, this type of virus is mainly used in

combination with Sf- or Hi-5 cells. The expression of recombinant proteins in the BEVS is under control of a very late viral promoter, often the strong polyhedrin promoter [14] or the p10 promoter [15]. The p10 promoter has been shown to be activated earlier in the infection time course than the polyhedrin promoter, which results in an earlier initiation of the expression of some recombinant proteins. Several systems use the BEVS in order to express the foreign gene under control of an immediate-early promoter (IE), which is active in uninfected cells as well as infected cells during the early infection phase. These systems have shown to be superior for the expression of eukaryotic secretory glycoproteins with high activity [16].

An elegant way to create recombinant Baculoviruses (BV's) is the bacmid technology. A bacmid is a shuttle vector between *Escherichia coli* and BV's. It replicates in *E. coli* and generates the recombinant viral DNA via site-specific transposition. After that, insect cells are transfected by the bacmid in order to produce the recombinant BV's [17]. The advantage of this method is the short development time necessary to generate the recombinant viruses. In addition, helper viruses are not required. BV's are not harmful to humans and due to the late expression also cytotoxic proteins can be produced. Disadvantages of the BEVS are the lysis of cells following infection as well as the proteolysis of recombinant proteins. Furthermore, the infection is an extra process step which often results in difficulties in reproducibility and process stability dependent on the quality of the virus stock. The heterogeneity of the infection affects the glycosylation pattern as well. Perfusion mode can be hardly performed with this system [18].

2.2.2 Stable Expression System

Virus infected cells have a finite life span. Therefore, untreated cells need to be infected in order to produce a fresh batch of recombinant protein. To overcome this limitation, non-lytic expression systems have been developed for insect cells as well. The stable expression system of insect cells is similar to that of mammalian cells. An expression vector carrying the gene of interest is stable integrated in the insect genome. The expression and secretion of the recombinant proteins requires no lysis of the cells. Traditionally, S2 cells were used with this type of expression system, but also for Sf- and Hi-5 cells stable expression systems are available. A wide variety of vectors allows the expression of recombinant proteins in S2 cells. For induced expression, a vector with the metallothionein promoter is used. Thereby the expression of the recombinant protein is induced with copper sulfate [19]. Constitutive expression of recombinant proteins in S2 cells is often from the actin 5 promoter [20]. More global stable expression systems for insect cells use the baculovirus-derived immediate-early OpIE promoters. These promoters originally derived from the Baculovirus *Orgyia pseudotsugata* multiple nucleopolyhedrovirus (*Op*MNPV) allow constitutive recombinant protein expression in a variety of cells types. These include Sf9, Sf21, Hi-5, S2, Kc and *Lymantria dispar* as well as mosquito cell lines. Both *Op*IE promoters (*Op*IE1 and *Op*IE2) provide relatively high levels of recombinant protein expression.

Nevertheless, the *Op*IE2 promoter has been shown to be up to 10-fold stronger than the *Op*IE1 promoter [21].

The stable expression system has several advantages. It allows a homogenous glycolysation profile of the recombinant protein and guarantees a higher reproducibility and process stability. Almost every cultivation mode can be used for protein production with this expression system. Due to the missing cell lysis during recombinant protein production, the contamination with host cell proteins is very low. This is superior for protein purification and avoids proteolysis of the target protein. The main disadvantage of this system is its time intensity compared to the BEVS, since the establishment of stable cell lines is a time consuming venture [18].

2.3 Comparison of the BEVS with Stable Insect Expression Systems

Several studies compared the BEVS with stable insect expression systems. Expression of two different proteins (extracellular vascular cell adhesion protein [VCAM], trans-membrane dopamine D4 receptor) was compared with the Baculovirus/Sf9 system and stable transfected Drosophila S2 cells. VCAM was produced in both systems in similar amounts and appeared identical within the framework of the performed analytics. D4 was also expressed similarly in both systems [22]. The expression of several antibody fragments was evaluated in the BEVS and in stable transfected S2 cells. Reported maximum concentrations of the recombinant antibody fragments were 9 mg/L in the BEVS and 0.4 mg/L in S2 cells [23]. Production of recombinant human IL-7 in the BEVS and stable transfected Sf cells revealed in 10 times higher expression levels for the BEVS [24]. Up to now the investigations on recombinant protein production do not show BEVS being superior to the stable insect expression systems. It strongly depends on the protein which is intended to be produced. For the choice of the best expression system not only the quantity of the recombinant protein is crucial, but also its activity which results from the right post-translational modifications.

2.4 Application of Insect Cell Based Expression Systems for the Production of Insect Derived Antimicrobial Peptides/Proteins (AMPs)

The use of insect cell based expression systems for the production of recombinant insect derived peptides/proteins is a relatively new field and emerged more and more as an attractive alternative to other commonly utilized expression systems. Especially for the production of insect derived proteins with a potential antimicrobial or antifungal activity, the insect cell based production systems can be a promising tool in order to provide satisfactory product yields and the specific

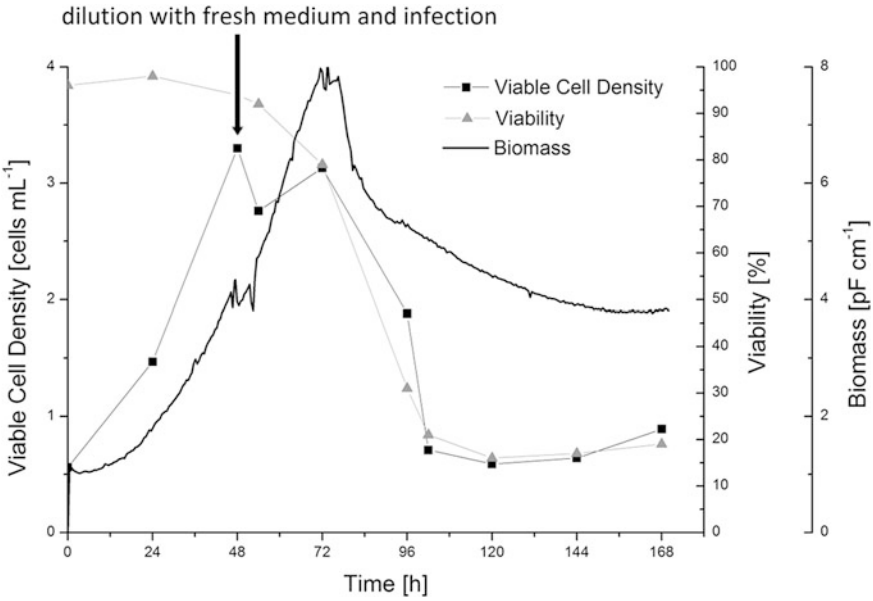

Fig. 2 Exemplary production process for *Gm*Glv-GFP in a 3 L bioreactor system using the BEVS with Sf21 insect cells, TOI $\sim 3 \times 10^6$ cells mL^{-1}, MOI = 0.1, cultivation temperature: 28 °C, dissolved oxygen concentration (dO_2): 40 % of air saturation, utilized medium: Sf-900 II SFM, the biomass signal is based on permittivity measurements via dielectric spectroscopy (see Sect. 3.8), viable cell densities were determined via trypan blue exclusion method

glycosylation patterns. The increasing demand for new AMPs is even more emphasized due to the increasing number of human pathogens showing resistance against available antibiotics. AMPs as novel anti-infective therapeutics could be an alternative to fight multiresistant bacteria strains. The lepidopteran model host *G. mellonella* has recently been reported as an attractive source for various AMPs such as gloverin, cecropin or the insect metalloproteinase inhibitor (IMPI) [25]. Gloverins are glycine-rich and heat stable basic proteins which primarily exhibit activity against gram-negative bacteria such as *E. coli*. [26–29]. However, also activity against gram-positive bacteria or fungi has been reported in the literature [30, 31]. The BEVS based expression of a *G. mellonella* derived gloverin, which has been fused to a green fluorescent protein for better protein detection (*Gm*Glv-GFP), represents a good example on how the protein itself might influence the production process. Figure 2 depicts an exemplary production of *Gm*Glv-GFP in a 3 L stirred tank bioreactor. Once the uninfected Sf21 insect cell culture has been diluted to approximately 3×10^6 cells mL^{-1} at 48 h cultivation time and simultaneously infected with the recombinant baculovirus at a multiplicity of infection (MOI) of 0.1, the cell density and permittivity based biomass (see Sect. 3.8) revealed the typical time course of a secondary infection dependent kinetic. The strong increase in biomass signal within the first 24 h post infection includes the typical swelling of Sf cells following baculovirus infection as well as cell growth.

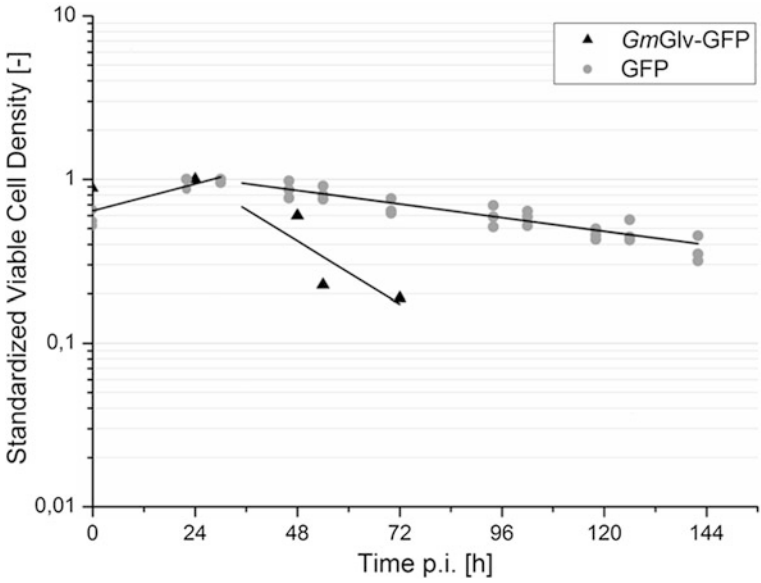

Fig. 3 Time courses for the standardized viable cell densities of BEVS-based production processes for *Gm*Glv-GFP and GFP in a 3 L bioreactor system, insect cell system: Sf21, baculovirus vector: AcMNPV, MOI = 0.1, TOI = $1 - 3 \times 10^6$ cells mL^{-1}, dissolved oxygen concentration (dO_2): 40 % of air saturation, utilized medium: Sf-900 II SFM, viable cell densities were determined via trypan blue exclusion method

After approximately 72 h cultivation time the biomass signal and cell density decreased as a result of cell lysis. A comparison of the cell death kinetic during *Gm*Glv-GFP production with the time course of cell death during expression of only GFP reveals a much faster dying of the cells for the *Gm*Glv-GFP production process (Fig. 3). For both baculovirus based production processes the same cell system (Sf21), the same virus (AcMNPV) as well as the same 3 L bioreactor system have been utilized.

Obviously the baculovirus induced *Gm*Glv-GFP production in Sf21 insect cells leads to distinctive cell stress, which finally results in a faster dying of the culture. Moreover, Moreno-Habel et al. [32] recently found that *Trichoplusia ni* derived gloverin (*Tn*Glv) leads to inactivation of budded baculoviruses (AcMNPV) by disrupting the viral envelop. An inactivation of budded baculoviruses affects infection kinetics and could lead to falsified infection doses as well as increased process variability. This finally can affect product titers. Typical product concentrations observed for *Gm*Glv-GFP produced in the BEVS range from 5 to 20 mg/L, which is quite low when compared to the theoretical productivity of the system. Gloverin as an example shows that the production of insect derived molecules in insect cells can be very challenging. On the one side the production of these molecules in insect cells is a logical consequence as it is very likely to produce an active molecule with 100 % correct folding and modifications. On the

other side it is possible that the recombinant molecule influences the production system either in a positive or, as observed for gloverin, in a negative manner. In this special case it has to be evaluated if gloverin only influences the baculovirus and sufficient product yields can be reached with the *Drosophila* S2 System, which works independent from viruses.

3 Online Monitoring

Modern bioprocesses demand for high efficiency, productivity and reproducibility in order to reduce costs and to ensure a satisfactory final product quality already during the ongoing production process. For that purpose, the Food and Drug Administration (FDA) launched the process analytical technology (PAT) initiative which is intended to achieve these goals by implementing modern process monitoring tools for critical process parameters during all stages of the production processes. Particularly in cell culture processes, small changes of critical parameters such as temperature, hydrodynamic conditions, pH, nutrient supply or dissolved oxygen concentration can lead to alternations in cell metabolism and finally influence product yield and quality [33]. Moreover, from the industrial point of view low process efficiencies and productivities can even result in unprofitability of processes [34]. Biopharmaceutical manufacturing processes still suffer from performances far below their theoretical potential since improvement is a time and cost consuming venture once the existing process has been approved by the regulatory authorities. Thus, real-time process monitoring of critical process parameters can allow for product definition already during the manufacturing process in order to avoid losses of production batches due to quality issues [35, 36]. Additionally, there is a growing need for bioprocess monitoring in disposable bioreactors which has been driven by the increasing demand for single use technologies in the biopharmaceutical industry [37]. Up to now, the connectivity of PAT-devices to disposable bioreactors is one of the major drawbacks [38].

In modern bioprocesses, monitoring of various parameters such as temperature, pH or dissolved oxygen concentration via common electrodes is already well established. However, there is still a strong demand for monitoring various other parameters of interest e.g. medium components, product concentration as well as cell density and viability, which allow a deeper understanding and insight into manufacturing processes [39]. Despite the increasing demand for a high and consistent product quality in the biopharmaceutical industry and the high potential for process improvement, available in situ monitoring techniques have been rarely established for insect cell culture processes. Due to the similarity of insect cell systems to other eukaryotic production systems, this contribution also includes application examples for various other animal as well as mammalian cell culture processes. Related in situ sensor concepts for the most common optical and capacitive monitoring techniques are introduced as well as their potential in monitoring cell culture processes and, if applicable, insect cell processes.

3.1 In Situ Analysis and its Requirements

Bioprocesses are usually sampled in order to gain information about parameters that may have a significant impact on culture performance. This finally facilitates a fundamental understanding and allows for process control in order to reach effective processing [38]. For instance, the concentrations of key substrates and, with respect to BV infected insect cell cultures, the time of infection (TOI) and time of harvest (TOH) are well known to influence product yield [40]. Gaining information about these parameters usually requires sampling from the bioreactor for offline analysis. However, major drawbacks of this approach are the time delay between sampling and analysis as well as the risk of contaminations [39]. In order to avoid these concerns, an in situ monitoring system can be applied to facilitate real time measurement directly in the bioreactor or in a bypass [41]. Bypass configurations are particularly applied in systems where, for instance, gas bubbles in the bioreactor would interfere the measurement signal [38]. However, for bypass-measurements it needs to be considered that within the bypass changes in state of the medium e.g. temperature, dissolved oxygen concentration, can cause falsified results when compared to measurements performed directly in the bioreactor [34]. Another possibility is to utilize the sensors externally (ex situ). In this case, a sterile sample removal system forwards the medium directly to the sensor (on line) [38, 42]. Since in situ sensors are placed directly in the sterile environment of the bioreactor, they have to fulfill special requirements. In situ sensors need to be autoclavable and should provide a stable and reliable signal over an extended period of time. This is especially the case when in situ sensors are applied for cell culture monitoring purposes since these processes are usually conducted over several days. A calibration previously performed has to be stable even after a sterilization cycle. Alternatively, there should be an option to calibrate the sensor after sterilization without sacrificing sterility of the system [38]. Moreover, disposable in situ sensors for single-use applications have to fulfill additional requirements. These sensors should be cheap enough to ensure economic efficiency, however, a long life time is not necessary. Technologies that could be mentioned in this context are semiconductor devices such as pH-ion-sensitive field-effect transistors (IS-FETs). These devices, for instance, can be installed directly into the cultivation medium to facilitate pH, temperature and dissolved oxygen analysis. Alternatively, it is possible to use non-disposable optical sensors in combination with transparent observation windows. So far, continuous sterile sampling devices for single-use applications which facilitate on line analysis via ex situ measurements are not commercially available [42]. Generally, the choice of which process analytical technology is necessary to monitor and control the bioprocess is also dependent on the employed biological system. While prokaryotic systems provide a fast increase in cell density, which comes along with a fast metabolic activity, eukaryotic systems only exhibit slower growth rates and, consequently, a slower metabolic activity. Therefore, eukaryotic

cultures require a lower recording frequency and analysis time for the sensor signal compared to prokaryotic systems [34, 38].

3.2 Infrared Spectroscopy

Spectroscopic methods such as the near infrared (NIR) spectroscopy and mid infrared (MIR) spectroscopy offer the advantage of monitoring various critical process parameters simultaneously. The function principle is based on the absorbance of energy and the resulting molecular vibrations and rotations once the related molecules have been excited at specific wavelengths. This energy absorption is finally identified and provides information about the molecules of interest [43]. The spectral signatures of organic molecules are thereby classified into three categories based on specific spectroscopic wave number ranges: far infrared (10–200 cm^{-1}), MIR (200–4,000 cm^{-1}), and NIR (4,000–13,000 cm^{-1}). Compared to the NIR signal, the MIR signal provides more defined peaks in the absorption spectra which facilitates a better assignment of molecules [39]. Especially the "fingerprint region" (500–1,500 cm^{-1}) in the MIR spectra allows for very specific identification of organic molecules since this region exhibits the most specific absorption patterns [38]. However, the applicability of this technique is limited due to the strong infrared absorption of water below wave numbers of 4,000 cm^{-1}. NIR spectroscopy has been reported to be a suitable tool for monitoring of substrates and products in biotechnology [38, 44]. Hydrogen bonds which exhibit distinctive stretch vibrations between 3,600–2,400 cm^{-1} allow for the detection of molecules that contain hydrogen bound to a heteroatom. Further potential analytes are proteins with their N–H bonds as well as O–H bonds of alcohols and C–H bonds of aliphates and aromates [39]. The possibility of utilizing IR spectroscopy for in situ applications in bioprocess monitoring was facilitated by the development of attenuated total reflectance (ATR) probes. A description of the measuring principle of ATR-IR spectroscopy is given by Lindner et al. [42]. Despite the fact that ATR-IR probes are commercially available, an application of the ATR-IR technology to disposable reactor systems remains a cost intensive venture since the expensive ATR crystals would have to be replaced after each cultivation process [42]. Furthermore, signal interpretation of multidimensional IR spectroscopic data is not straightforward and requires chemometric techniques. These techniques usually include data reduction via principal component analysis (PCA) as well as the development of calibration models via multivariate regression methods such as principal component regression (PCR) or partial least square regression (PLS). Also artificial neural networks (ANN) can be applied, especially when the correlation between the spectral data and target variables exhibit distinctive non linearity [36].Various authors utilized IR spectroscopic techniques in cell culture applications. The most applications of NIR and MIR spectroscopy have been performed to monitor glucose, lactate as well as glutamine and ammonia in Chinese hamster ovary (CHO) cell culture processes [45–47]. Beside

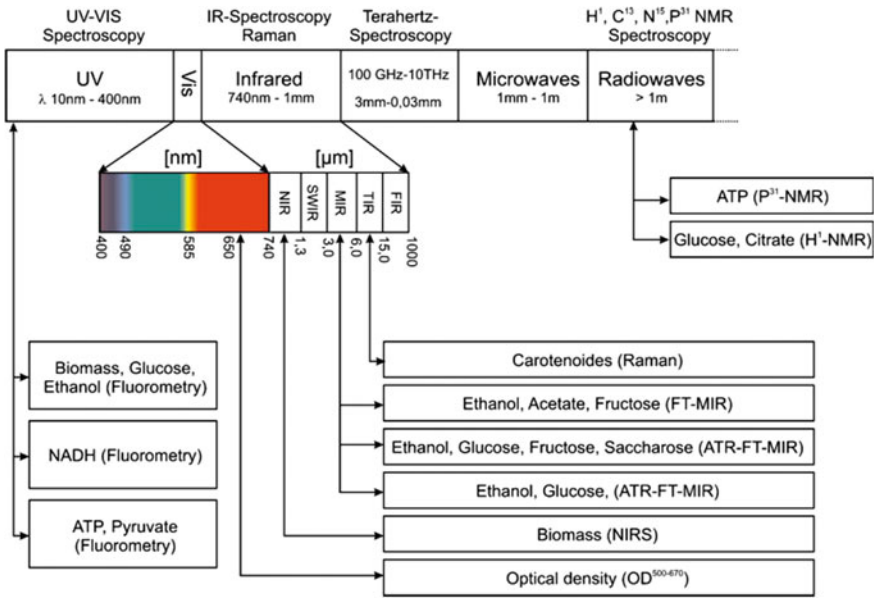

Fig. 4 Electromagnetic wavelength spectrum and the corresponding wave length ranges for various optical and spectroscopic sensor techniques (Beutel et al. [38]; with kind permission from Springer Science and Business Media)

glucose, lactate and ammonia, Henriques et al. [48] also employed NIR spectroscopy to determine cell density in mammalian cell culture processes for monoclonal antibody production. Sellick et al. [49] simultaneously predicted glucose, lactate as well as monoclonal antibody concentrations in supernatants of CHO and murine myeloma (NS0) cell cultures via fourier-transform (FT)-MIR spectroscopy. An application of NIR spectroscopy for in situ monitoring of glucose and lactate during bioreactor cultures of adherent Vero cells attached to microcarriers is reported by Petiot et al. [50]. Only Riley et al. [51] applied off-line NIR spectroscopy to monitor the glucose and glutamine concentrations during growth of Sf-9 insect cells cultivated in Sf-900 II serum-free media. Despite the complexity of the culture media, glutamine and glucose concentrations could be predicted with standard errors of 0.51 and 1.46 mM, respectively, indicating the feasibility of IR-spectroscopic techniques in monitoring insect cell culture processes (Fig. 4).

3.3 Fluorometry

Direct measurement of fluorescence in an in situ application represents a promising tool for bioprocess optimization since various biologically active substances such as amino acids, enzymes, cofactors and vitamins exhibit fluorescent activity when

excited at specific wave lengths. First applications of fluorometry for monitoring purposes was limited to only one pair of excitation and emission wavelength, and thus, to a single fluorophore [36]. In these applications, the nicotinamide adenine dinucleotide NADH and its phosphorylated form NADPH has been the fluorophore of choice since the intracellular NAD(P)H concentrations could be utilized to estimate biomass concentrations [52]. However, major drawbacks of this technology are the dependency of the fluorescence signal to environmental conditions such as pH or temperature as well as the possibility for overlapping signals caused by the presence of other medium components fluorescing at the same wavelengths as the component of interest [34, 36, 53]. Further concerns that have been described to decrease fluorescence yield are inner filter effects, cascade effects and quenching phenomena [36, 54, 55]. Therefore, multivariate chemometric techniques are necessary to enable reliable data interpretation. In contrast to the single wavelength fluorometers, 2D fluorometers allow the detection of multiple fluorophores at once. The principle is based on the application of various excitation and emission wavelengths resulting in a complete fluorescence spectrum [34, 36, 38]. However, fluorometry has only scarcely been applied in monitoring eukaryotic cell culture processes. A possible reason might be the complexity of the culture media. In mammalian culture media, for instance, various fluorescent amino acids need to be provided since mammalian cells are not capable of synthesizing these amino acids on their own [56]. Once these amino acids are incorporated into recombinant or cellular proteins, their fluorescent properties might change. For instance, electrostatic interactions of tryptophan with neighbored charged amino acid residues of e.g. glutamate, lysine or aspartate, can lead to a significant shift in the emission wavelength of tryptophan [57]. This and other phenomena, such as quenching effects, make direct correlations between the growth of mammalian cells and fluorescence signals a difficult task [36]. Anders et al. [58] utilized in situ fluorometry to measure the NAD(P)H dependent culture fluorescence in uninfected and infected Sf-9 insect cell cultures. The fluorescence signal correlated well with the total cell density. A decrease in the fluorescence signal could be detected as a result of the decrease in viable cell density due to nutrient depletion. Comparable experimental results were obtained in serum containing Grace and TC-100 insect cell medium as well as serum free Ex-Cell 401. In mammalian myeloma NSO cell culture, Hisinger et al. [59] demonstrated the use of 2D fluorescence spectroscopy for monitoring the GFP concentration during a GFP production process. Teixeira et al. [56, 60] applied traditional 2D fluorescence spectroscopy as well as synchronous fluorescence spectroscopy for monitoring the production of recombinant glycoprotein IgG1-IL2 and monoclonal antibody IgG4 in Baby Hamster Kidney (BHK) and CHO cell culture processes, respectively. Besides product formation, they also demonstrated the suitability of these techniques for monitoring viable cell densities. Especially the synchronous fluorescence spectroscopy provides the potential for bioprocess monitoring in a real-time context since this technique exhibits improved peak resolution and recording speed [60].

3.4 Raman Spectroscopy

The principle of Raman spectroscopy is based on shifted wavelength scattering of molecules due to inelastic collisions of photons with the molecules once excited with monochromatic light. Bioprocess applications of Raman spectroscopy are feasible since adjustable lasers are available. The technique can be applied for multi-analyte measurements as well as differentiated measurements of certain compounds [61]. Resulting molecular fingerprints are well defined with high chemical specificity even in aqueous systems [62]. Despite these advantageous, Raman spectroscopy has not been extensively utilized for in situ applications in cell culture processes, which might be related to the difficulties arising with the use of this technique. One of the main problems is the considerable fluorescence activity of several biological molecules that might overlay the Raman scattering bonds [60, 61, 63]. This is even more a problem when the fluorescent compounds cannot be eliminated, as is the case for culture broths. A common approach to overcome this issue is the use of wavelengths outside the excitation range of the fluorescing molecules. Another problem arising from the use of charge-coupled device (CCD) detectors, which might exhibit pixel-to-pixel sensitivity, is the appearance of stable variations. Magnitudes of these variations can even exceed magnitudes of the measured signals [38]. Especially in bioprocess applications, bubbles from aeration and biomass can cause light scattering which results in signal attenuation. In this case, internal referencing can be utilized for correction purposes [61]. The high potential of using Raman spectroscopy in monitoring of cell culture processes was successfully demonstrated by Abu-Absi et al. [62]. In this work, Raman spectroscopy was employed to monitor glucose, glutamine, glutamate, lactate, ammonium, as well as the viable and total cell density in 500 L CHO cell culture processes. Chemometric techniques have been applied to the training datasets in order to obtain the required calibration models. Especially the possibility of monitoring the cell viability, which can be calculated from the predicted viable and total cell densities, represents an additional advantage.

3.5 Optical Chemosensors for O_2, pH and CO_2 Determination

The application of optical chemosensors (optodes) facilitates measurements of parameters not accessible via direct measurements. Measuring principles of these sensors are based on indicators with optical properties such as photoluminescence, reflection and absorption. Available concepts are suitable for common bioreactors as well as disposable systems. The externally placed optical detector unit can be interfaced to the transducer via optical fibers [42]. O_2, CO_2 and pH can be measured in a non-invasive manner from outside the bioreactor via transparent observation window. Expendable sensor patches, containing the immobilized indicator, are thereby placed inside the bioreactors. Alternatively, invasive

measurements are possible via probes immersed in the culture broth and equipped with an optical window and indicator spot at the tip. This set up allows the application of optodes even in stainless steel bioreactors without optical windows. Compared to the classical electrodes, optical chemosensors represent a promising alternative for conventional bioreactors as well as disposable systems and can easily be implemented in cell culture processes. For instance, optical oxygen sensors have been successfully applied for oxygen monitoring in human mesenchymal stem cell cultures in fixed bed bioreactor systems [64–69].

3.5.1 Optical O_2 Sensors

Optical oxygen sensors are based on fluorescence quenching by molecular oxygen [70, 71]. After excitation with light e.g. by a light emitting diode (LED), the lifetime and intensity of a fluorescence signal is proportional to the oxygen concentration present around the dye. Emitted fluorescence light is transmitted via the optical fiber and segregated from the reflected excitation light by a dichroic mirror. Subsequent fluorescence detection is achieved by a photodiode or photomultiplier. Optical oxygen sensors are autoclavable without loss of sensitivity [42]. Compared to conventional Clark electrodes, commercially available O_2 optodes (e.g. available at PreSens, Regensburg, Germany/Ocean Optics Inc., Dunedin, FL, USA) are calibration-free and do not require polarization time or replacement of the electrolyte solution prior to use. A major drawback that needs to be mentioned is the limitation of long-term stability caused by photobleaching [42]. Therefore, the sensor patches need to be replaced on a regular basis.

3.5.2 Optical CO_2 Sensors

CO_2 sensors have been based on potentiometric function principles such as the Severinghouse electrodes [72]. These sensors are equipped with a pH and a reference electrode surrounded by a hydrophobic CO_2 permeable membrane. The electrodes are thereby placed within a carbonate buffer. When CO_2 concentration outside the permeable membrane increases, CO_2 molecules diffuse into the carbonate buffer and change the pH value, which finally can be described by the Henderson-Hasselbalch equation. Fiber-optic CO_2 optodes are based on a similar pH-sensing system, where the electrodes are replaced by a pH-sensitive dye [73]. Compared to these classical CO_2 optodes, the development of solid type optical CO_2 sensors, with ion-pairs directly immobilized in the membrane, resulted in faster response times as well as a reduced sensitivity to ionic strength [74]. Determination of CO_2 via optical sensors can either be based on absorbance or fluorescence. Intensity based quantification methods have been the preferred ones since problems associated with the short life time of the fluorescence signal increased the need for sophisticated instrumentation. A method to overcome this drawback is based on the conversion of the intensity signal or the luminescence

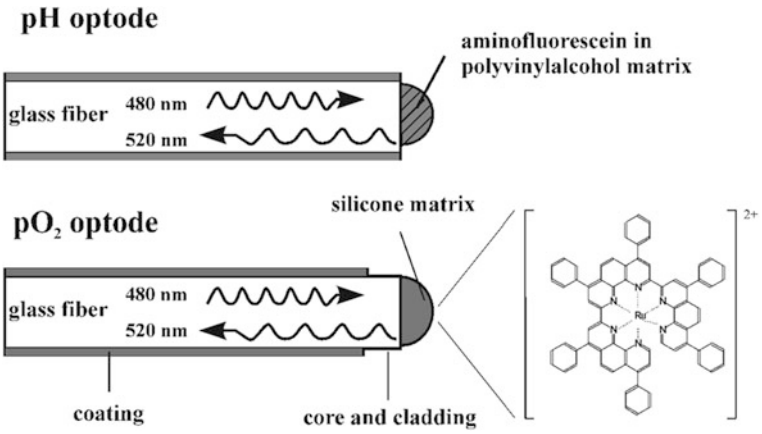

Fig. 5 Working principle of optical pH and pO_2 chemosensors (Glindkamp et al. [80]; with kind permission from Springer Science and Business Media)

decay time of a pH-sensitive dye into a long-lifetime signal via resonance energy transfer (RET) [73].

3.5.3 Optical pH Sensors

Measurements of the pH via optical sensors can either be based on absorbance or fluorescence indicator dyes [75]. Today, pH can be measured in a range between pH 1–11 [76]. Also the cross-sensitivity to ionic strength for fluorescence based pH sensors, which has been reported as one of the drawbacks [77], could be reduced by the use of esterified fluorescein derivatives [78]. Covalently bound fluorescent dyes are suitable to decrease the loss of sensitivity due to elution of the dye or temperature treatment during autoclaving [79] (Fig. 5).

3.6 In Situ Microscopy

In situ microscopy (ISM) is a promising tool for bioprocess monitoring in cell culture applications since the technology offers real time information about various parameters such as cell concentration, cell size distribution or cell morphology. The probe is thereby directly immersed in the culture broth. Images taken by the CCD-camera are analyzed by sophisticated image analysis algorithms. Generally, in situ microscopes can be subdivided into two different groups, the incident light microscopes with an optically defined analysis zone and the transmitted light microscopes containing an optically or mechanically defined analysis zone [81, 82]. However, in the studies published so far only the transmitted light in situ microscopes have been applied for cell culture monitoring purposes. A transmitted

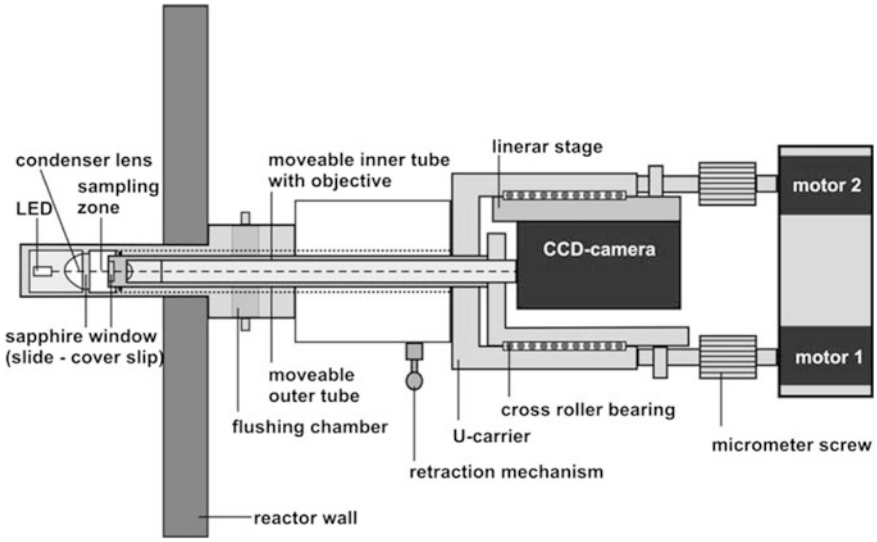

Fig. 6 Construction scheme of an in situ microscope developed by Frerichs and Joeris (Höpfner et al. [81]; with kind permission from Springer Science and Business Media)

light in situ microscope that meets the demands for industrial applications was described by Frerichs and Jöris in 2002 [83, 84] (Fig. 6).

In order to allow the adaption to changing process conditions, this microscope contains a mechanical defined variable-volume flow-through sampling zone. The sampling volume can be adjusted by a sampling zone tube which is connected to a movable slide. Beside the sampling zone tube, the microscope also contains two additional tubes, an outer probe tube as well as an inner objective tube. Proper focusing of the image is facilitated by the inner objective tube mounted to a second movable slide. Both slides are connected to a U-shaped profile and can be controlled by two separate stepper motors or micrometer screws. Illumination of the sampling zone via LED provides sufficient light for the visualization of cells by the CCD-camera. Since the reactor segment of the microscope can be separated from the optical segment, the sensor can be sterilized in the autoclave. The microscope has been employed for the monitoring of CHO and BHK cell cultures as well as adherent mouse fibroblast cells (NIH-3T3) on microcarriers [81, 83–86]. In order to recognize cells attached to the surface of microcarriers, the optical density of the microcarriers turned out to be an important parameter. Cytodex 1 microcarriers were found to be suitable for microscopic cell observations and image analysis due to the low optical density of the polydextrin matrix. Especially the reported change in the greyscale distribution between microcarriers without cells and overgrown with cells was assumed to be applicable for an estimation of cell densities [85]. In this context, Rudolph et al. [86] demonstrated the suitability of different greyscale

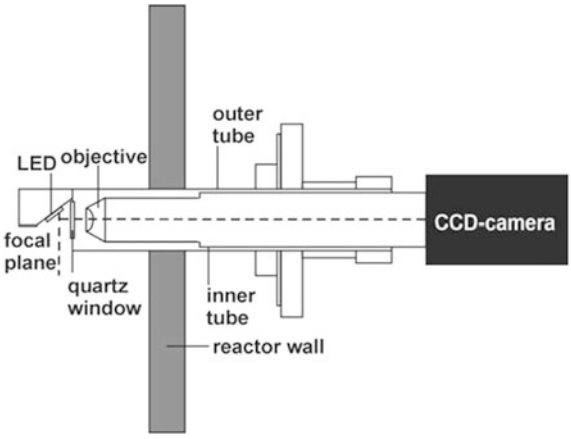

Fig. 7 Construction scheme of a transmitted light in situ microscope equipped with an optically defined sampling zone (Höpfner et al. [81]; with kind permission from Springer Science and Business Media)

distributions in order to determine the planting efficiency and level of colonization during cultivation of NIH-3T3 cells on Cytodex 1 microcarriers.

The application of a transmitted light in situ microscope with optically defined sampling zone utilized to monitor BALB/c hybridoma cells was presented by Guez et al. [87]. The microscope described in this study is based on a pulsed illumination of the sampling zone via LED, which is synchronized with the image generation of the CCD-camera. This setup facilitates image generation of moving cells without motion blur. The LED is thereby mounted to an outer tube angularly to an optical quartz window. In order to facilitate proper focusing, the microscope contains a movable inner tube equipped with a 40× magnification objective. Since the microscope does not enclose a defined sample volume mechanically, information about the cell concentration is derived from a virtually defined volume (*depth from focus*—procedure [88]) (Fig. 7).

Beside information regarding cell density, cell size distribution and cell morphology, the development of an in situ dark field microscope also facilitates measurement of the cell viability. The dark field microscope developed by Wei et al. [89, 90] is based on the transmitted light in situ microscope described by Frerichs and Jöris in 2002 [83, 84]. For the purpose of dark field microscopy, the illumination of the original microscope has been modified. The original condenser was replaced by a dark field condenser. The application of Support Vector Machine (SVM) classifiers, which have been trained by a dataset of images with either living or dead cells, facilitates an automated determination of cell densities and viabilities. So far, the technology was only applied for fermentations of *Saccharomyces cerevisiae*, but also represents a promising method in cell culture applications. Another important point is the applicability of in situ microscopy in disposable systems. Up to know the use of ISM in disposable systems is not examined, but different approaches are already envisioned in the literature [42].

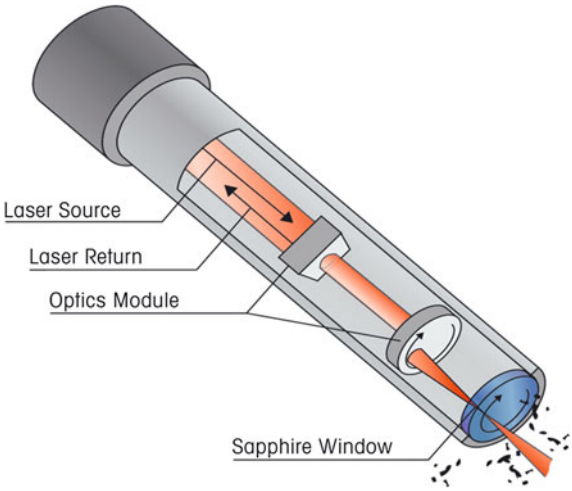

Fig. 8 Layout of a FBRM probe (with kind permission from Mettler-Toledo)

3.7 Focused Beam Reflectance Measurement (FBRM)

The FBRM technology as an in situ monitoring tool for cell culture processes offers the potential to gain real-time information about various important process variables such as cell density, cell size distribution as well as cell morphology. Inside the FBRM system, a laser light generated by a laser diode is passed to the probe assembly via fiber optics. The probe, which can be immersed in the culture broth, contains optical components that transfer the incoming laser light to an eccentrically rotating lens. The rotating lens finally facilitates a constant circular movement of the laser beam (2 m/s) as well as a focusing of the laser light to a small spot. As the laser beam intersects particles passing through the measurement zone, light is backscattered towards the probe. The backscattered light is then collected by the optics and forwarded to a photo diode, where the optical signal gets detected [91] (Fig. 8).

The duration of reflection multiplied by the rotation velocity of the laser beam finally results in a chord length, which considerably depends on the particle size as well as the particle shape. Compared to the rotation velocity of the laser, the velocity of the particles passing through the measurement zone can be neglected. The measurement range is 1–1,000 μm and the measured particles are sorted into different channels according to measured chord lengths, resulting in a characteristic chord length distribution. Thus, the technique does not deliver the particle size distribution directly. Empirical or theoretical methods are applicable in order to determine the particle sizes from the chord length data [92–94]. Although the probe has already been utilized for offline characterization of microbial [95–98] and plant cell systems [99–101], applications in cell culture processes are not yet reported. The latest version of the G400 FBRM system distributed by Mettler-Toledo is fully autoclavable and therefore allows the integration of this technology

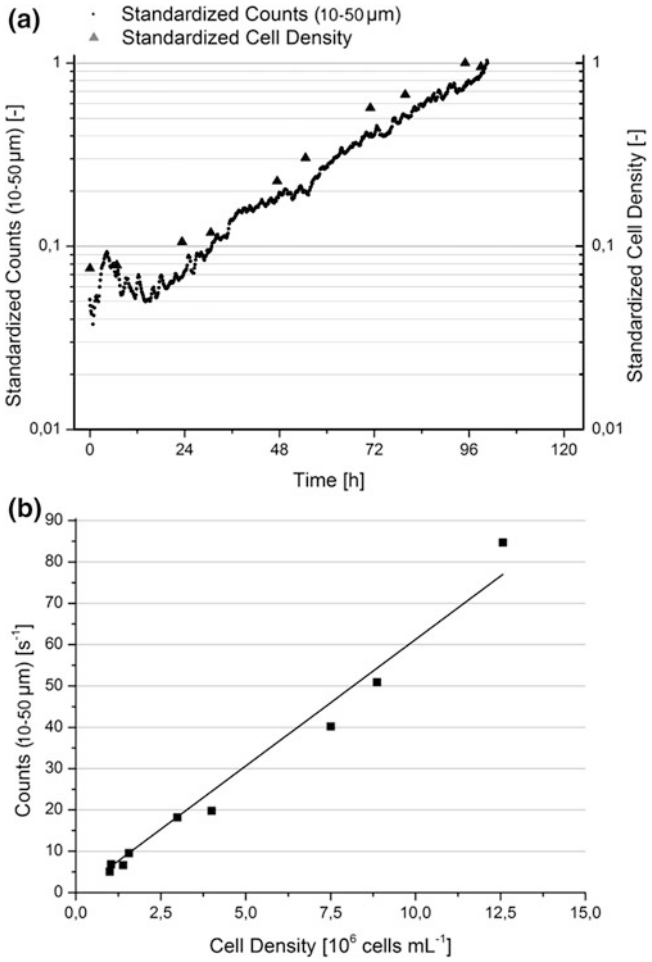

Fig. 9 **a** Standardized FBRM counts in the range of 10–50 µm and the corresponding standardized offline cell density for a *Drosophila* S2 insect cell culture process; **b** Linear correlation between the FBRM counts (10–50 µm) and the offline total cell density for a *Drosophila* S2 insect cell culture process

even in cell culture processes. As indicated in Fig. 9, the system provides reliable information regarding the exponential growth of *Drosophila* S2 insect cells cultivated in a sparged and agitated 1L bioreactor system. Air bubbles generated by the O-shaped sparger did not interfere with the FBRM cell counts. The measured amount of cell counts per second in the chord length range of 10–50 µm exhibited a linear correlation with the offline total cell densities and reflected the exponential growth of the cells. Further research needs to be done in order to examine whether this technology can be utilized to follow the changing mean cell diameter after baculoviral infection of insect cells as observed for the *S. frugiperda* derived cell

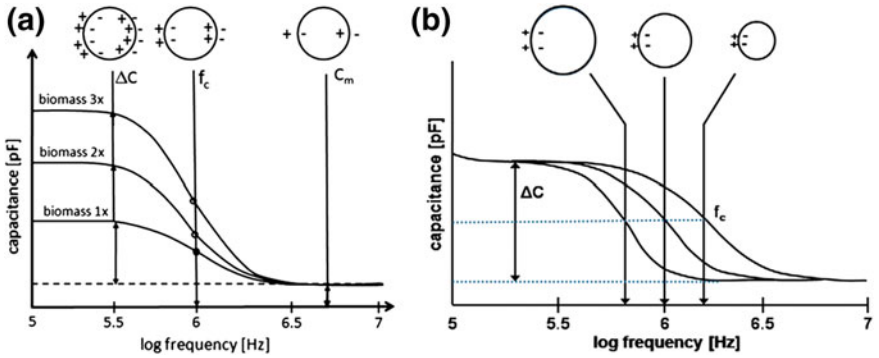

Fig. 10 **a** β-dispersion spectra for increasing cell densities with the capacitance ΔC critical frequency f_c, and medium capacitance $C_{m;}$ **b** β-dispersion spectra for changing cell sizes, the indicated variation in the critical frequency f_c is valid for a constant biovolume (Cannizzaro et al. [106]; with kind permission from John Wiley and Sons)

lines Sf9 and Sf21. In this case, the change in mean cell diameter would result in a corresponding variation of the mean chord length. As a result, the mean chord length signal could be utilized to evaluate a successful infection of the cells.

3.8 Dielectric Spectroscopy

The so called dielectric or impedance spectroscopy sensors belong to the group of capacitance and conductivity sensors. The technology is based on the passive dielectric properties of e.g. cells in a conducting medium. Beside the surrounding culture medium, the cytoplasm of the cells also contains conductive solutes such as salts and nutrients. Due to the dielectric properties of the lipid-based cell membrane, electrically charged ions accumulate at these cell membranes when an alternating electric field is applied to the cell suspension. In this case, the cell membranes act as small capacitors leading to a buildup of electrical charge (polarization). The measured overall capacitance is thereby dependent on the applied frequency of the alternating electric field, which is usually in the range between 0.1–10 MHz, as well as the cell size and cell concentration. At low electric field frequencies the ions have sufficient time to reach and polarize the cell membranes leading to a high overall capacitance of the cell suspension. With increasing excitation frequencies the polarization and thus the capacitance of the cell membranes decreases. In this case, the ions do not have enough time to move and accumulate at the cell membranes before the electric field changes direction. The observed drop in the measured capacitance from low to high frequencies exhibits a sigmoid shape which is also known as the β-dispersion spectrum [102]. As indicated in Fig. 10, the β-dispersion contains the critical frequency f_C which represents the working point for impedance sensors in the frequency range

mentioned above. Since the polarization is dependent on the ability of the cell membranes to stop ion movement, only cells with intact cell membranes are involved in the buildup of the capacitance signal. Thus, dead or leaking cells are not captured by this technology [103]. Additionally, the capacitance signal is reported to be insensitive to gas bubbles and microcarriers facilitating the monitoring of cell culture processes in common sparged as well as fixed bed bioreactors [102, 104, 105]. However, very high volume fractions of non-biomass materials close to the sensor may influence the capacitance signal since the polarizable cells are replaced by non-polarizable materials [102].

The increasing demand for disposable cultivation systems emphasized the development of compatible sensors. Today, several solutions for the application of dielectric spectroscopy in single use bioreactors are available from the manufacturers (Aber Instruments, Aberystwyth, UK and Fogale nanotech, Nimes, France) [42, 107].

Dielectric spectroscopy offers a great potential in monitoring insect cell culture processes, especially the baculovirus related production processes, since the arrest of cell growth after infection as well as the swelling and lysis of infected cells can be monitored in real-time. The first online monitoring of infected and uninfected Sf9 insect cell cultures using dielectric spectroscopy was reported by Zeiser et al. [108]. The obtained results for uninfected insect cells indicated a linear correlation between the relative permittivity and the viable cell density during growth. After synchronous infection with a recombinant baculovirus encoding for β-galactosidase (MOI = 10), which resulted in an arrest of cell growth, the permittivity signal further increased as a result of the increasing cell size. Finally, the signal coincided well with the decrease in cell viability and size during cell lysis in the late infection phase. In order to enhance the β-galactosidase yield, the same work group focused on infection and feeding strategies and demonstrated the use of dielectric spectroscopy for monitoring high cell density cultivations of High-5 [109] as well as Sf9 insect cells [109, 110]. In either case, physiological parameters correlated well with the impedance signal. An observed peak in the CO_2 evolution rate during Sf9 cultivation could be related to a temporary plateau in the relative permittivity signal, reflecting the onset of the release of virus particles into the culture broth. However, this signal plateau has not been detected in the case of lower MOI infections of 0.001. In this case, the missing simultaneous cessation of cell growth resulted in a delay of the CO_2 evolution rates [109]. Furthermore, the results indicated that further optimization and automation of the fed batch processes potentially could be based on the permittivity signal [110]. Negrete et al. [111] employed dielectric spectroscopy in order to analyze and characterize the production of recombinant adeno-associated vectors (rAAV) in a 40 L tank using the baculovirus expression vector system with Sf9 insect cells. The permittivity signal allowed a determination of the infection time since the viable cell density and growth rate of non-infected cells could be monitored in real-time. Moreover, the increase in cell diameter could be correlated to the yield of rAAV as well as the optimum harvest time (TOH). Ansorge et al. [112] monitored infected Sf9 cell cultures and found a good correlation between the permittivity signal and the

viable biovolume (determined by Vi-CELL®) as well as the total biovolume (determined by CASY® 1). After infection, the characteristic (critical) frequency f_C was observed to change according to the changing cell diameter. However, impact of the cell membrane properties on f_C could not be excluded. Besides the monitoring of insect cell cultures, dielectric spectroscopy has been extensively utilized to monitor mammalian cell cultures such as hybridoma, CHO, Vero or HeLA cultures. These applications have already been reviewed by other authors [107, 113] (Table 1).

4 Scale Up of Industrial Insect Cell Culture Processes

4.1 The Baculovirus Expression Vector System for Commercial Vaccine Production

The Baculovirus Expression Vector System (BEVS) is nowadays considered an established and matured manufacturing technology for the commercial production of a wide array of recombinant proteins in an industrial scale, e.g. of virus like particles (VLP) for vaccines [114]. As of mid-2012, a total of five human or veterinary vaccine products based on the BEVS system have been approved. Examples include GSK's CERVARIX® (Human Papillomavirus) and Boehringer Ingelheim's CircoFLEX® (Porcine Circovirus Type 2). Several other products are already in development and BEVS-based vaccines are considered for more and more diseases, including for the field of emerging diseases. Recent literature provides a good overview over the current state of commercial production utilizing the BEVS [114, 115].

4.2 Process Scale Up and Implications for Processes Utilizing the Baculovirus Expression Vector System

The BEVS production system requires the growth of an insect cell line, preferably in suspension culture, and its infection with a specific recombinant BV carrying the genetic information for the protein of interest. The infection can either be carried out subsequently to cell growth or simultaneously with cell planting into a vessel. For the commercial vaccine production it is crucial to establish an efficient, cost-effective and robust large-scale process to manufacture the required quantities of the protein of interest [115]. Taking the process developed initially in the milliliter or liter scale and scaling it up to industrial production volumes of hundreds or thousands of liters is a challenging procedure. Maintaining the productivity of the small scale process as well as critical process and product quality attributes are the essential goals of the scale up procedure [116–118].

Table 1 Summarized applications of monitoring techniques in insect cell cultures

Cell line	Technology	Application	Reference
Sf9	NIR Spectroscopy	Monitoring of glucose and glutamine in insect cell culture samples (offline)	[51]
Sf9	Fluorometry	In situ monitoring of NAD(P)H dependent culture fluorescence in infected and uninfected cultures; correlation with cell density	[58]
Sf9	Dielectric Spectroscopy	In situ monitoring of infected and uninfected cultures; correlation with viable cell density during growth; signal increase due to increasing cell size after infection	[108]
Sf9; High-5	Dielectric Spectroscopy	In situ monitoring of high cell density cultivations up to 50×10^6 cells/mL; detection of virus release after simultaneous infection	[109]
Sf9	Dielectric Spectroscopy	In situ monitoring of fed-batch processes for β-galactosidase production; increase in permittivity after infection at 14×10^6 cells/mL could be attributed to cell size increase	[110]
Sf9	Dielectric Spectroscopy	Characterization of a rAAV production process in a 40L large-scale vessel; permittivity signal allowed a determination of the infection and harvest time	[111]
Sf9	Dielectric Spectroscopy	In situ monitoring of cell cultures during growth; change in critical frequency f_c following infection was assumed due to changes in cell size and membrane properties	[112]

A well developed, optimized and characterized small scale process is essential for a successful scale up [119]. Implementation of strategies like Quality by Design (QbD) or Process Analytical Technology (PAT) and the utilization of high throughput systems help to achieve these goals by increasing the process understanding, monitoring and control. The use of suspension insect cell lines readily facilitates the possibility of large scale processes as required for the commercial production of vaccine proteins and it is probably safe to assume that most commercial BEVS processes are performed in stirred tank reactors (STR). Biological process parameters of importance in the BEVS system are generally considered to include cell density at infection, multiplicity of infection, time of infection and physiological state and age of the cells at the time of infection [115]. But engineering parameters like agitation rate (mixing and shear), dissolved oxygen concentration or pH are also considered to be of significant importance for the process.

Cell culture condition, and therefore eventually the cells physiological state, affects and determines product quantity as well as quality. Scale up of suspension culture systems like the BEVS and its related equipment is performed based on well understood principles of scaling relevant parameters. Often it is based on geometric similarity of the small and large scale vessels [120]. In such a case, one or more specific parameters are then kept constant from the small to the large scale. Literature provides a good overview about the potential criteria for scale up [116]. They include, but are not limited to, power input, impeller tip speed or shear rate, Reynolds Number Re, oxygen transfer coefficient, gas flow rate per unit volume, mixing time and similar. It should be noted that because some of these criteria are partially associated with each other, keeping one of them constant can result in a change of the other parameters during scale up, in turn causing a change in the physical environment the cells experience [121]. Nevertheless, geometric similarity is often not given, for example when transferring or scaling up an established process into production facilities where equipment is already installed or if equipment is designed as multi-purpose equipment. Also, single use systems may not always be of the same geometry as the small scale or the stainless steel STR currently used. In such a case the process and its control need to be modified accordingly. In any case, the equipment used obviously needs to meet the current regulatory as well as process specific requirements. More recently, the application of Computational Fluid Dynamics (CFD) has also become more popular in the field of bioprocess scale up [116].

Volumetric scalability is considered as one of the key benefits of the BEVS systems, indicating the relative ease with such a system can be scaled up to commercial scales [122, 123]. Several key aspects need to be considered when scaling up a BEVS based process from the laboratory to large scale. Generally speaking, system heterogeneity increases with increasing scale, which has some important implications for the scale up procedure. Sufficient mixing via impellers has to be ensured at the large scale to allow sufficient mass transfer and to avoid major nutrient, oxygen or pH concentration gradients in the culture. Impeller agitation related shear stress has long been thought to be a major issue for (not only) insect cell cultures, but nowadays is not considered a major issue anymore.

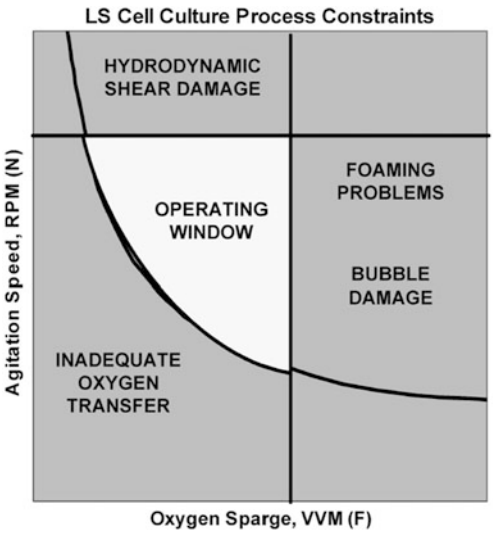

Fig. 11 Large scale cell culture process constraints (Marks [125]; with kind permission from Springer Science and Business Media)

The use of multiple and supposedly shear sensitive impellers as well as medium additives like Pluronic F68 further helps to reduce the impact of this type of physical damage [118, 124]. Similarly nowadays it should not be a problem anymore to achieve the required oxygen transfer rates to supply the insect culture with sufficient oxygen. But even while the oxygen demand of insect cell cultures is relatively low compared to aerobic bacterial processes, surface aeration is generally not sufficient at the large scale and sparging of air or oxygen is therefore usually required [124, 125]. This leads to the problems of bubble induced cell damage as well as potential foaming issues which require the selection of the correct sparger type as well as gas composition and flow rates. Bubble size is also an important parameter to consider. Smaller bubbles, for example generated by sintered spargers, can cause more cell damage in insect cell cultures than large bubbles [124, 125]. Bubble size also affects CO_2 removal from the culture which can be considered another significant parameter to evaluate during scale up of a BEVS process [126]. Overall, an acceptable equilibrium between the parameters in question has to be found for the specific process and equipment used (see Fig. 11).

4.3 Single-Use Systems in Baculovirus Expression Vector System Processes

In recent years single use systems like the Single-Use Bioreactor (HyClone) or WAVE® (GE Healthcare) have been become more and more utilized in the biotech industry in general but also in BEVS processes [127, 128]. Main advantages of single use systems are their reduction in cross contamination potential as well as cleaning costs, increase of flexibility and decrease of the turnaround time. Main

disadvantages on the other hand are the risk of leachables or extractables, sometimes insufficient material strength and difficulty of handling, pressure as well as temperature limitations, increased costs of waste disposal and scale limitations [115, 129]. Regardless of the specific system employed, the basic principles for scale up and transfer of a BEVS process into such a system are the same as for the standard stainless steel stirred tank equipment.

5 Conclusions

For the production of biologically active recombinant proteins the insect cell expression system is considered to be a suitable alternative to bacterial or mammalian cells. Compared to bacterial fermentations recombinant protein production in insect cells produce fully post-translational modified proteins. This is also possible with mammalian cells but is much faster and easier to implement with insect cells. BV expression vectors are commonly used in combination with lepidopteran species such as *S. frugiperda* (Sf9 and its parental line Sf21). However, infection of Sf9 cells by the baculovirus leads to cell lysis and a consequent dying of the culture. Here, the use of stably transfected *D. melanogaster* S2 cells can be a promising alternative. In contrast to the BV induced protein production in Sf9 cells, S2 cells are not lysed during cultivation facilitating even continuous process modes and protein production at high cell densities. Comparisons of the BEVS with the stable S2 expression system regarding protein yields clearly indicate that neither of the systems can be assumed superior. The amount of protein produced in both systems as well as process performance strongly depends on the properties of the protein itself. This has been further demonstrated using the example of a production process for *Gm*Glv-GFP. In order to choose the right expression system, not only protein yields should be considered but also the quality of the proteins resulting from post-translational modifications.

In situ technologies suitable to provide real-time information regarding nutrient consumption, product and metabolite formation, cell morphology, cell size, cell density and even cell viability, result in a deeper understanding of cell culture processes and allow the early detection of potentially unfavorable changes during production processes that might have a negative impact on product formation and quality. Hence, in situ monitoring contributes to meet the "quality by design" (QbD) requirements imposed by the FDA, resulting in a high and consistent product quality in the biopharmaceutical industry. Moreover, in situ monitoring reduces the risk for contaminations caused by common sampling procedures and represents an ideal tool for process control and automation. Despite the increasing demand for monitoring techniques in disposable systems, commercially available solutions which facilitate the connection of optical in situ sensors to disposable reactors are still rare. However, various methods are already described in the literature. Monitoring techniques such as the dielectric spectroscopy, in situ microscopy or FBRM are suitable to provide information on cell responses after

viral infection making these technologies interesting for the optimization of baculovirus related production systems. In this context, interrelations of critical process parameters such as the MOI, TOI and TOH can be evaluated. The high potential of optical monitoring techniques to determine nutrient and metabolite concentrations for optimization purposes in cell culture processes have not been fully exhausted yet. However, due to the fast developments in the area of in situ online monitoring systems driven by the PAT initiative and the increasing demands for high quality biopharmaceuticals, further applications of these technologies can be expected in the near future.

Also in industrial large scale processes, online monitoring techniques are intended to implement QbD strategies by increasing the process understanding and control. The use of suspension insect cell lines enables production processes in common stirred tank reactors. This is beneficial from the industrial point of view since stirred tank reactors can be used as multi-purpose equipment. However, in order to transfer small scale insect cell production processes into production scale, various aspects need to be considered. These aspects include biological parameters as well as engineering parameters. Geometric similarity is often not given especially when equipment is already installed and used as multi-purpose equipment. Moreover, disposable systems may differ significantly from small scale production vessels. In order to maintain culture performance, the process and the belonging process control needs to be modified in a way that regulatory as well as process specific requirements are considered. Finally, these goals can only be achieved by finding a compromise that ensures satisfactory yields of high quality products as well as the implementation of equipment specific requirements.

Up to now process optimization and process understanding for insect cell based processes is still immature. Some process engineering principles can be adopted from bacterial and mammalian fermentations which particularly are intensively investigated since decades. Nevertheless, the biological reaction of the insect cells to process changes cannot be simulated but can only be experimentally determined. Same is true for online monitoring of insect cells where mammalian cell processes can only give suggestions. The transferability of the online monitoring technology to insect cell processes still needs intensive research. Summarizing, insect cell processes still provide room for improvement to further increase the maximum yields of active recombinant proteins.

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Insect Antenna-Based Biosensors for In Situ Detection of Volatiles

Matthias Schott, Christoph Wehrenfennig, Tina Gasch and Andreas Vilcinskas

Abstract Insect antennae are among the most sensitive and selective chemical-sensing organs in the animal kingdom. Insects can perceive picograms of specific volatile organic compounds per cubic meter of air in milliseconds, which is far below the detection thresholds of current analytical devices. These exceptional sensing abilities have many uses in the context of insect biotechnology. Living specimens or parts of them, such as isolated antennae or individual proteins, can serve as biosensors in the field. As volatiles occur in a crude mixture in the environment, knowing which trigger-volatiles are crucial for the insects' perception of specific incidents is of great value. This knowledge promotes the development of selective sensors for applications, such as fire detection. In this chapter, we discuss the different technical procedures for the preparation and use of insect-based biosensors for the detection of organic volatiles, including those based on insect behavior, insect olfactory proteins, and biomimetic sensing units. We also consider the use of these applications in portable devices outside the laboratory under field conditions.

Keywords Biosensor · Electroantennography · GC–MS/EAD · Mobile GC–MS · Needle-trap device · Odorant receptor · VOC detection · VOC quantification

Abbreviations

EAG	Electroantennography
EAD	Electroantennographic detection
FETs	Field-effect transistors
FID	Flame ionization detector

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GC	Gas-chromatography
MS	Mass-spectrometry
NTD	Needle-trap device
OBPs	Odorant-binding proteins
ORs	Odorant-receptors
POM	Polyoxymethylen
SSRs	Single-sensillum recordings
SPME	Solid-phase microextraction
VOCs	Volatile organic compounds

Contents

1 Introduction

The sensitive and specific detection of volatile organic compounds (VOCs) is required in a wide range of technical applications, such as non-invasive diagnostics, process monitoring, quality assurance, detection of taints and off-flavors in the food industry, agricultural and environmental monitoring, and detection of explosives, drugs, and biological warfare agents [1, 2].

Insects are the most diverse taxonomic class in the animal kingdom, and they have evolved specialized sensory organs in their antennae for the detection of volatiles (i.e. semiochemicals, also known as infochemicals) that are used to communicate between individuals, including pheromones for intraspecific communication and allelochemicals for interspecific communication [3]. Insects use self-produced pheromones and environmental volatiles for navigation, communication (including deception), and the identification of nutrient sources. This has been reinforced by communication not only among insects but also across taxonomic kingdoms (e.g. interactions between insects and plants). The production of communication volatiles is an energy-demanding process; thus, the lowest possible

amounts are released to conserve energy and ensure that enemies cannot read the signals. When a signal is detected, a specific perception ensures that energy-consuming false reactions are avoided. The sensory organs of insects have therefore become particularly sensitive and specific, providing an ideal basis for the development of biosensors. Insects can sense odors at detection thresholds that are orders of magnitude lower than the most advanced physical approaches based on gas sensors or gas chromatography (GC)-mass spectrometry (MS) [1]. The olfactory system of insects is so selective that isomers of the same substance can appear as attractants or repellents depending on their spatial configuration [4]. There are several examples of isomer-specific reception in insects; for example, the gypsy moth (*Lymantria dispar*) and the nun moth (*Lymantria monacha*) are attracted by (+)−disparlure, but the gypsy moth is repelled by (−)−disparlure [5, 6].

The number of different perceivable volatiles differs between insect species, depending on their mode of life. On one hand, generalists such as the honeybee (*Apis mellifera*) are known to feed on many different plants and live for several months in changing environmental conditions. On the other, there are specialists that live for a very short time as imagos and never feed, with mating before death as the only goal. This is the case for specimens of the mayfly genus *Ephemeroptera* and several moth species. Some of these males not only sense the sex pheromone blend of the female but also volatiles from plants that are ideal mating sites, thus further increasing the likelihood of finding a female. Host plant volatiles can also promote mating behavior (e.g. in the European grapevine moth *Lobesia botrana*) to ensure that mating is favored at good feeding sites for larvae [7]. The extreme capabilities of insect antennae can be used in several ways, ranging from the use of whole insects to single olfactory proteins as sensors, including “copy nature” strategies in which antennae are used as templates for sensor systems. In each of these methods, the odorant–receptor–ligand interaction must be converted into a readable signal [1].

2 Principles of Insect Volatile Reception

To understand the advantages and disadvantages of different sensing concepts, we must first consider the cellular events involved in volatile detection. Insects have a diverse morphology that extends to their antennae. The distribution of sensilla (organs containing ORs) over the antennal and palp surfaces can be investigated with a light microscope [4], but the variation in the type and form of the sensilla [8] must be analyzed by scanning electron microscopy (Fig. 1). Different sensillum types (e.g. mechanoreceptors, thermoreceptors, hygroreceptors) fulfill specific tasks and occur in diverse contexts. There are also chemoreceptors for tasting (single-pore sensilla) or smelling (multi-pore sensilla). The scanning electron microscope image of the basal part of the sensilla in Fig. 1e shows a perforation covering the entire surface, representing pore tubules that enable volatiles to reach the sensilla lymph (Fig. 1f). Here, odorant binding proteins (OBPs) attach to the

perceivable substance to make hydrophobic volatiles soluble and to protect the

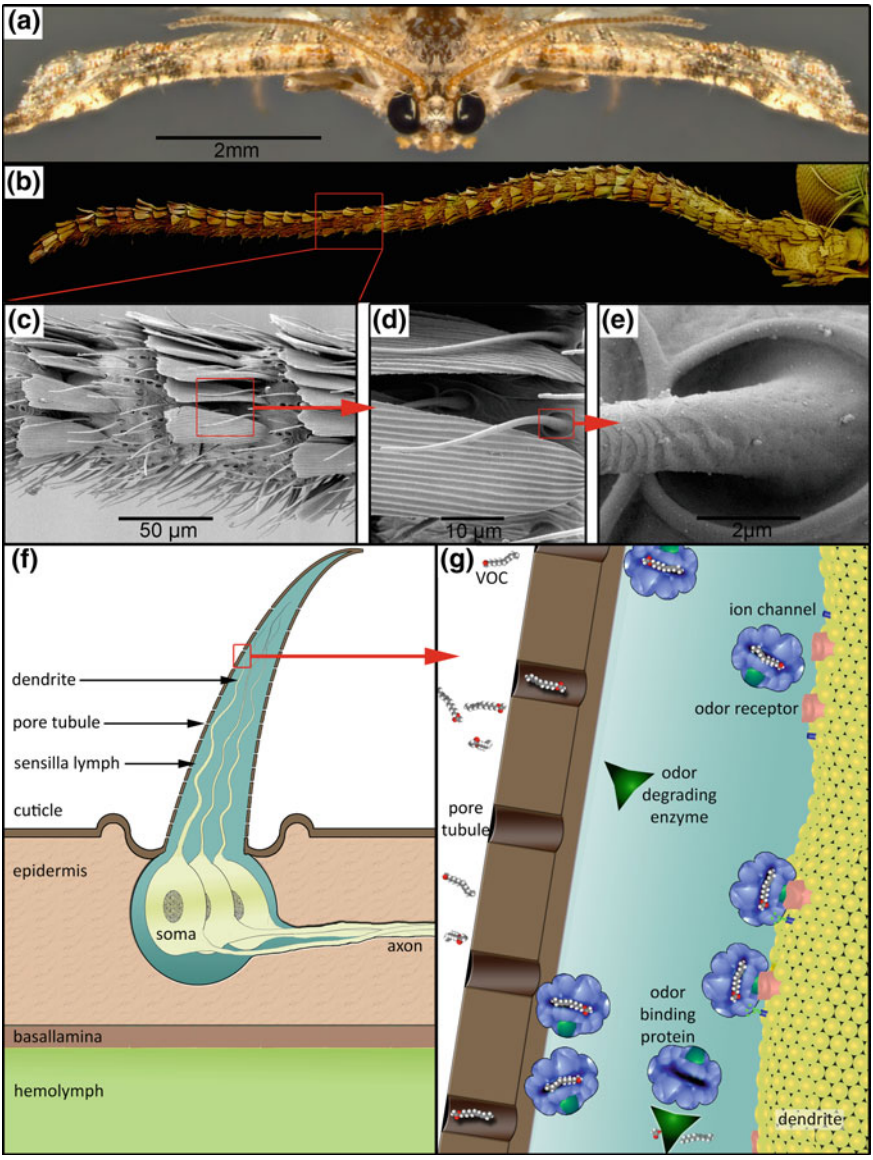

Fig. 1 **a** *Lobesia botrana* male. **b** Combined and pseudocolored scanning electron microscopy images of the *L. botrana* antenna. **c–e** Magnification of sensilla with pore tubule openings visible on the sensillum surface in **e**. **f** Sensillum structure, with the receptor neurons consisting of dendrite, soma and axon that reach to the antennal lobe (*after* [4, 8]). For clarity, the supporting trichogen, tormogen and theogen cells are not shown. **g** Volatile reception at the dendrite membrane with the associated proteins and enzymes (*after* [9])

volatiles from odorant-degrading enzymes (Fig. 1g). OBPs that carry a volatile can bind to membrane-bound odorant receptors (ORs) or release the volatile at the receptor surface so the volatile can bind directly to the receptor [9]. In either case, binding initiates a chain reaction that is still incompletely understood [1]. Ultimately, ion channels or ion-channel-like chaperones are opened. If enough odorants are present and therefore several ion channels have opened simultaneously, an action potential is released along the dendrite to the antennal lobe, although the underlying mechanisms again remain elusive [10].

Subsequently, the volatile is released and degraded by a specific odorant-degrading enzyme to prevent further signals being triggered by the same molecule. Most sensilla contain 1–5 different dendrites [8]. Exceptions include the hymenopteran *Sceliphon spirifex*, which has 140 olfactory receptor neurons per sensillum [8, 11]. Moths in particular tend to possess pheromone receptors that recognize only one specific compound, so that even minimal chemical variations elicit no reaction even in high doses at these specific receptors [12]. By contrast, general ORs can also react to several substances as especially reported in flies, and the subsequent perception of single odorants is achieved by pattern recognition, as in *Drosophila melanogaster* [12]. Therefore, the specificity of the devices based on insect reception depends on the species and the target volatiles.

3 Electroantennographic Devices

3.1 Laboratory Systems

3.1.1 Electroantennography (EAG)

The most widely used and established method to measure insect volatile reception is electroantennography (EAG, see Fig. 2), as it was first reported in 1955 by Schneider [13, 14]. In such devices, insect antennae are connected to two electrodes and electrical signals are measured when substances are received. The sum of depolarization events on the dendrite surface (Fig. 1g) generates a nanovoltage that must be filtered and amplified to measure the signal. The electrodes can be connected to the insect tissue via saline solutions (e.g. KOH or Insect Ringer's solution) or gels used for electrocardiograms. They prevent dehydration and rapid healing of the injured insect tissues, therefore enabling the measurement of antennal signals.

EAG measurements are also possible with dissected heads or whole insects, in which case the measurement electrode is connected to the antennal tip and an indifferent electrode to the antennal suture or insect neck [15, 16]. Whole insects must be immobilized to prevent false signals from motor neurons. The placement of the reference electrode is important to prevent the measurement of signals from other nerves. Whole insect measurements are beneficial in species in which the

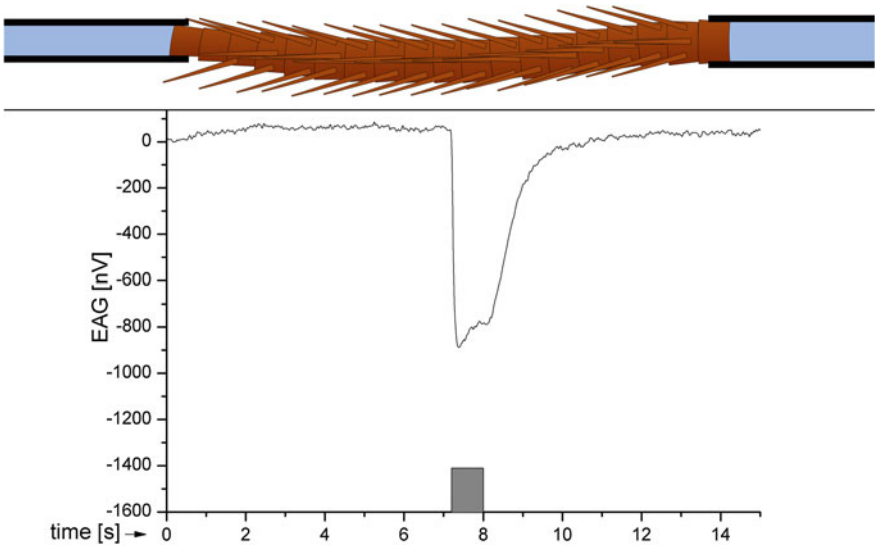

Fig. 2 Typical electroantennography (EAG) connection and measurement. An isolated insect antenna is inserted into a glass electrode filled with Insect Ringer's solution. The graph shows an EAG recording, with an air puff that contains a test compound presented for one second (as indicated by the *gray bar*). The EAG response features a negative potential that correlates with the sum of all depolarization events in the antennal receptors. The example represents a *Lobesia botrana* male antenna that reacts to an air puff above a filter paper spiked with 100 pg 7,9-dodecadienyl acetate diluted in acetone

antennae survive only briefly after isolation. The classic EAG setup also works underwater; for example, antenna from the water beetle *Dytiscus marginalis* are reported as equally sensitive in water and air [17].

In a standard EAG setup, a steady stream of cleaned and humidified air is presented to the insect antenna to achieve a standardized background for the mechanoreceptors, hygroreceptors, and thermoreceptors. One puff of air containing a specific concentration of a test substance is then added for a fixed period of time (up to 1 s). Before each experiment, the blank reaction of the antenna to the clean air puff must be recorded to evaluate the reactions of the sensilla towards electromagnetic fields in the apparatus. However, the responsiveness of the antenna decreases over time. Therefore, to compare the measurements taken from one antenna, reference reactions must be recorded at regular intervals so that values can be normalized afterwards [18, 19]. Several methods are used to feed test substances into the air stream, often involving a defined amount of substance placed on a permeable filter paper, on a rubber septum, or in a drop of paraffin oil [18, 20, 21].

The major drawback of EAG is that purest substances are required for accurate recordings because the reactions of all antennal neurons are measured at the same time. In some cases, it is difficult to obtain a pure or synthetic reference substance. Isomerism must also be considered when interpreting the results.

3.1.2 Coupled Gas Chromatograph-Electroantennographic Detection (GC-EAD)

To circumvent the disadvantages of EAG, it is possible to separate blends of volatiles by GC. When the EAG is used as detection unit after this separation, the apparatus is termed as a coupled gas chromatograph–electroantennographic detector (GC-EAD) [22]. Different chromatographic columns and temperature/pressure programs allow the separation of almost all types of volatile compounds. The addition of a flame ionization detector (FID) also allows quantitation, and a MS helps to identify the compounds that are separated. FID and EAD devices can be exchanged because both work under atmospheric pressure, but the integration of a mass spectrometer is more difficult because the GC column ends here in a high vacuum. To enable simultaneous measurement of substances eluting from the gas chromatograph, a column coupling combined with makeup gas is required to compensate for pressure differences. The combination of GC and EAG allows active compounds to be separated from mixtures [22], such as the reaction of a *L. botrana* male antenna to the enrichment of outside air near a pheromone dispenser containing *L. botrana* female pheromone components as shown in Fig. 3. By analyzing the isomer mixture of *Z,E*, *E,Z*, *Z,Z* and *E,E*-7,9-dodecadienyl acetate, Arn et al. [22] proved that *L. botrana* male antenna respond mostly to the *E,Z*-isomer, the main component of the pheromone blend, while showing little response to the *Z,E*-isomer. In several insect species such as the gypsy moth, the incorrect isomers can even have a negative effect [5, 6].

GC-EAD offers the unique possibility to quantify substances based on the antennal reaction. As the response to the preparation differs from one antenna to another, it is necessary to normalize the data. Provided that the measurement setup ensures constant temperature, air flow, and humidity, the responses are

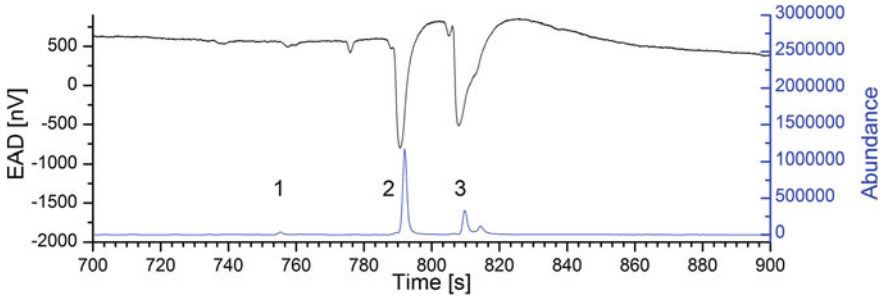

Fig. 3 Comparison of electroantennographic detection (EAD) and mass spectrometry (MS) responses in a coupled gas chromatography-mass spectrometry/electroantennographic detection (GC-MS/EAD) measurement of a needle-trap device enrichment near a pheromone dispenser in a vineyard. (1) Butylated hydrotoluene (antioxidant added to the pheromone dispensers). (2) 9-dodecenyl acetate (major pheromone component of *Eupoecilia ambiguella*). (3) Two diastereomers of 7,9-dodecadienyl acetate (major pheromone component of *Lobesia botrana*). The *upper trace* displays the EAD response of a *L. botrana* male antenna and the *lower trace* shows the abundance of ions measured by MS [23]

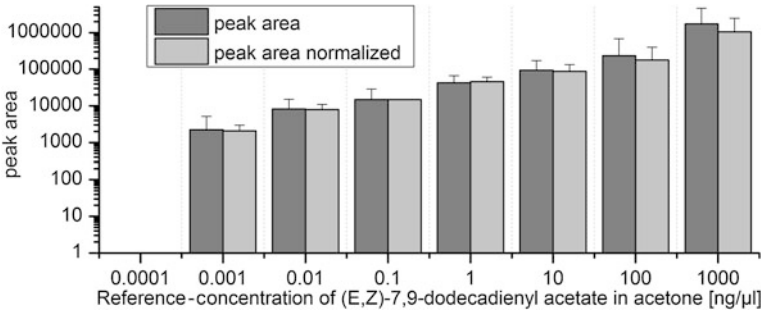

Fig. 4 Dose-response peak areas of *Lobesia botrana* males to 1 µl injections of (*E,Z*)-7,9-dodecadienyl acetate in acetone measured using gas chromatography-electroantennographic detection. *Dark bars* show antennal responses from 1 pg to 1 µg and *error bars* indicate the standard deviation. *Dark bars* represent the measured peak areas and *light bars* show the reaction after normalization to 0.1 ng/µl (adapted from [23])

reproducible because antennae from the same species feature the same calibration slopes [22–24]. Normalization to one specific concentration injected into the GC injector and measured with each antenna ensures the repeatability of measurements with different antennae (Fig. 4).

The most recent development is a chopper-modulated EAD, which promises to lower the detection limit even further. Here, the signal-containing air flow is mixed with clean air at a specific frequency and "chopped" into blocks with and without target volatiles. The reaction of the antenna to the air stream therefore oscillates at the same frequency. A matched band pass filter can then be used so that only the reaction to the target substances is passed while the noise from the other receptors and the electrical device is filtered out. This approach has increased the sensitivity of the used EAD by a factor of 10^4 [25, 26].

3.1.3 Single-Sensillum Recording (SSR)

Recordings with whole antenna or whole insects are not selective for single volatiles and do not reveal which sensilla are responding to each specific substance, but single-sensillum recording (SSR) does provide this information. Here, an extremely narrow glass capillary or tungsten electrode is connected to the tip of a sensillum or inserted in the cuticle near a sensillum pit. The grounding electrode is connected to the cut base of the antenna, the intersegmental membrane, the eye, or abdominal tip to establish contact with the hemolymph [29–31] (Fig. 5). The advantage of this type of measurement is that every depolarization event can be measured as a spike, resulting in highly accurate surveys and specific measurements. However, the amplitude of each signal is lower than the measurement of the summed depolarization events recorded by EAG. Therefore, the whole measurement setup is even

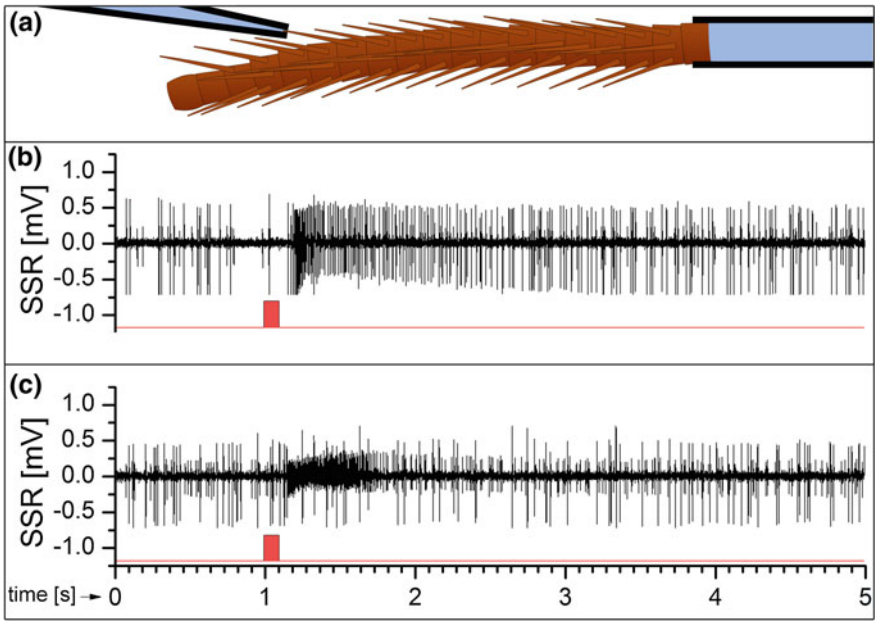

Fig. 5 A single-sensillum connection and two typical single-sensillum recordings (SSRs). **a** The connection of the sensillum tip and the antennal base to glass capillaries filled with Insect Ringer's solution. **b–c** Recordings of a *Drosophila melanogaster* ab3-sensillum that comprises two olfactory receptor neurons [27]. The larger cell contains Or22a receptors that respond to ethyl hexanoate (**b**), whereas the smaller cell contains Or85b receptors that respond to 2-heptanone (**c**). In both traces, the cells were presented with 100-ms pulses of 10^{-4} dilutions of each compound in hexanoate, indicated by the *gray box* (for details, see [28]). The SSR response displays greater spike density representing the depolarization events on the respective dendrite surface, in the presence of each specific compound

more susceptible to electromagnetic fields and tremors. For this reason, the SSR apparatus is typically shock proof and mounted in a Faraday cage. In SSR, the preparation is especially challenging, as can be seen from the sensillum size in Fig. 1. Because there are normally only up to five different olfactory receptor neurons in one sensillum, the measurement is much more selective. But the type of sensillum cannot be determined from its shape under a light microscope in most species and classification can be hampered further when the sensilla are housed in a pit [8]. After connecting to a potential target sensillum, testing must be carried out with a pure test substance to exclude mechanoreceptors, thermoreceptors, hygroreceptors, and irrelevant types of chemoreceptors [32]. A GC-SSR coupling is a powerful tool to identify the ORs in one sensillum and the active substances in a multicomponent pheromone mixture [33].

3.2 Portable Systems

3.2.1 Portable EAG

By 1990, several portable EAG systems were under development [34–36], ultimately yielding a commercial device [37]. *In situ* EAG measurements with portable devices have been reported in vineyards, cotton fields, pea fields, apple orchards, forests, cranberry bogs, and maize plots [38]. In these assemblies, an insect antenna is mounted in a holder chip (Fig. 6a) made from an inert plastic such as poly(methyl methacrylate), also known as Plexiglas or polyoxymethylene (POM). The chip comprises two wells containing Insect Ringer's solution and an electrode. A narrow slit between these wells is bridged by the dissected insect antenna, which is exposed to the volatile-containing air. The chip is mounted in a glass or plastic chamber. In this approach, diverse chips offer the utilization of different antennal morphologies. The chip is beneficial because it resists minor shocks while its portability allows the rapid preparation of antennae away from the instrument. This facilitates the preparation process because conventional EAG devices are not that readily accessible. The researcher—a major source of tremors and electromagnetic fields—can also stay away and minimize the distortion. The volatile-laden air is forced through the measurement chamber and past the antenna by a suction pump (Fig. 6b). The external air must be cleaned by a charcoal filter (for calibration), which can be opened automatically to measure reactions to the volatiles in the air surrounding the device. A chemical reference is also required to calibrate each antenna, allowing the results from individual antennae to be compared and to monitor the decline in antennal response due to aging. The calibration solution has to be a pure chemical substance, which is the focus of the survey, and has to be dissolved in a solvent that does not stimulate an antennal reaction itself. Air puffs are then passed through gas-tight syringes that contain the chemical standard diluted in paraffin [36], in solvent absorbed onto filter paper [39] or in silicone oil [21]. The air puffs are emitted by motor-driven syringes into the air-mixing tube that leads to the EAG chamber described above (Fig. 6b). A Pasteur pipette containing filter paper spiked with 1 µl of calibration solution can also be used for calibration [14, 16]. Air, which is controlled by an electric valve, is puffed through the Pasteur pipette into the mixing tube that leads both clean and calibration air past the antenna. Results of outside measurements can be obtained in a few seconds from the portable EAG device, allowing relative atmospheric pheromone concentrations to be determined [41]. The device is also relatively small, so it can be carried around and placed at different heights to investigate the three-dimensional structures of pheromone plumes.

One disadvantage of this device is the reaction of the antenna to nontarget volatiles and environmental changes, such as plant-emitted volatiles or changes in temperature and humidity. Several calibration syringes with standards of nontarget volatiles can be used to blank out the background, saturating the respective olfactory receptor neurons to reduce or eliminate this problem [20]. However,

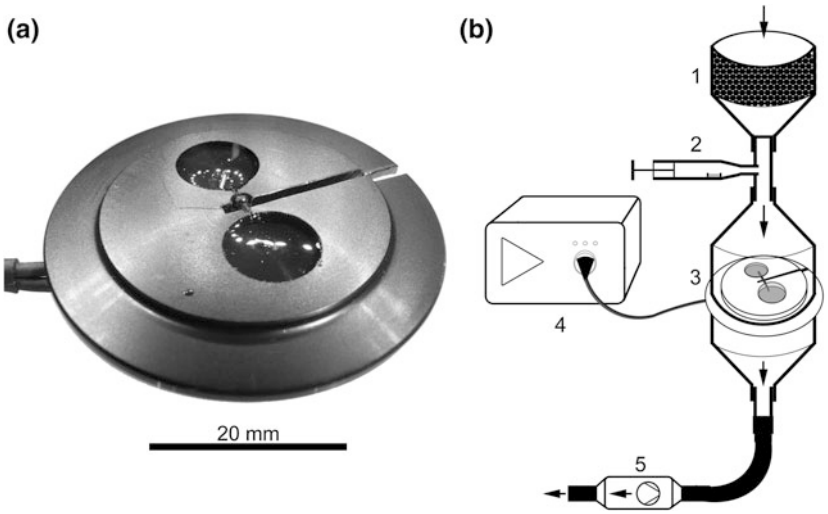

Fig. 6 **a** A self-built polyoxymethylene (POM) chip, which holds the antenna, with two wells each containing Insect Ringer's solution and an electrode separated by a slit. A *Nicrophorus vespilloides* antenna is placed in the slit for measurement (Schott et al. unpublished; adapted from [36, 40]). **b** A portable EAG device (adapted from [36]). Air is sucked in through a removable active coal filter (1). The filter is attached during calibration but can be removed for outside measurements. The antenna can be tested and calibrated using a syringe that provides air puffs containing calibration solution (2). The impulses from the antenna must be amplified and converted into a digital signal, which is recorded by a computer (4). The air is forced into the device by a suction pump (5)

every perceivable volatile at the measurement site must be known and available as reference, and the background volatiles may vary with the season [39]. Furthermore, the lifetime of the antenna is reduced because the background volatiles induce action potentials in the corresponding dendrites, thus consuming the limited available energy. Changes in temperature and humidity can also generate false positives/negatives.

Problematic background volatiles can be addressed using EAG-based bioelectronic sensor arrays as described by Myrick et al. [42]. These comprise up to eight antennae from different insect species and sexes, which are used simultaneously. Substances can be identified by pattern recognition and background peaks can be detected if the system is "trained" with specific volatiles. Such arrays require also insect antenna that do not detect the target volatile so that background reactions to environmental changes can be recorded. Mechanoreceptors, thermoreceptors, and hygroreceptors within each antenna will elicit signals when the environmental conditions change. Even so, absolute pheromone concentrations and comparable data are difficult to obtain with a portable EAG device; thus, quantitative measurements cannot be achieved [39].

Vickers et al. [43] developed the first artificial insect-mounted EAG to measure pheromone flux in a wind tunnel during the flight of a moth. A measurement

antenna was mounted between the antennae on the head of a living moth (*Heliothis viricens*), allowing data to be recorded during flight towards a pheromone plume. However, this setup only provides reliable data in a closed environment with constant temperature and humidity.

3.2.2 Portable EAD

To prevent false positives induced by plant volatiles and to quantify target volatiles, it is necessary to separate these substances in the field and shield antennae from outside conditions. Small and portable versions of the GC-EAD, including small helium or hydrogen gas bottles, are used to establish measurements in field. The portable EAD also allows the quantification of volatiles *in situ* and the measurement of absolute concentrations, because the antennal reaction can be compared to standard solutions. Calibration using standard air puffs with a calibration solution is necessary to monitor the ageing of the antenna, so it is possible to obtain several measurements with a single antenna. Under optimal conditions, dissected antennae can operate for up to 10 h, whereas in the field for up to 3 h [23].

Gas chromatography dilutes test substances in the carrier and make-up gases, so the volatiles must be enriched from the outside air for the antenna to respond to low concentrations of volatiles. Dynamic headspace enrichment (e.g. using a needle-trap device [44]) accelerates the enrichment time compared to still air enrichment (e.g. solid-phase microextraction). Accurate quantification requires that the enrichment method does not allow breakthrough (substances escaping the trapping material during enrichment) and achieves optimal recovery (all trapped substances desorb in the GC injector). This can be tested by connecting two enrichment devices in series. If target volatiles are detected in the second device after enrichment, a breakthrough has occurred. Optimal recovery is achieved if no residues can be detected in the enrichment material from the first device after desorption [44]. In portable GC-EAD measurements, the chip that holds the antenna as discussed above offers advantages compared to mounting the antenna in glass capillaries, especially if measurements are taken in windy regions. With the chip, it is possible to adjust the antennae in a sheltered place and transport it to the actual measurement side. During field measurements in vineyards, it was possible to measure the target volatile after 1 h of enrichment with a NTD-GC-MS/EAD (Fig. 7; [23]). Here, the portable GC–MS-EAD also allowed the identification of plant volatiles and the possible determination of their impact.

The major advantage of a portable GC-EAD is the ability to calibrate each antenna by injecting a defined standard solution. Hence, measurements from different antennae can be compared to determine absolute concentrations, even if the measurements take place in different climatic conditions [23].

Figure 8 shows an antennal trace aligned with the corresponding mass spectrum. The antennal trace shows reactions to the perceivable (*E,Z*)-7,9-dodecadienyl acetate (main component of *L. botrana* pheromone), whereas the MS trace shows reactions to 100 pg/µl but not 10 pg/µl of pheromone solution. This

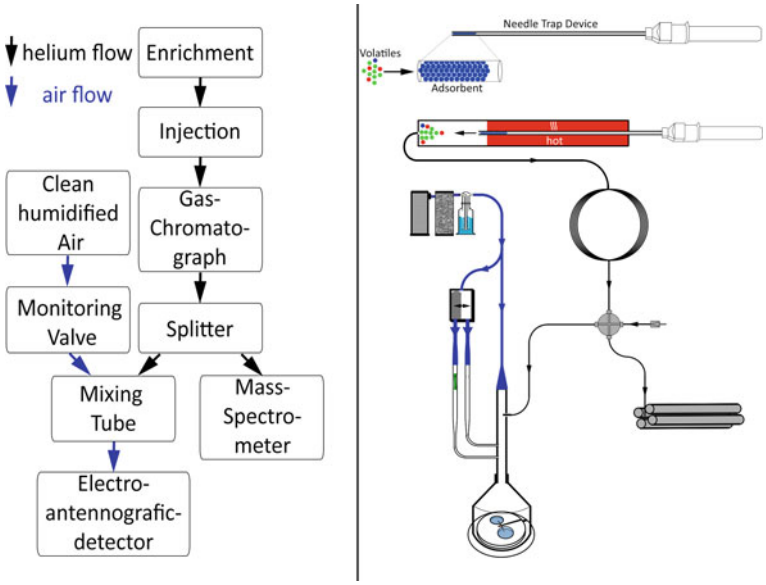

Fig. 7 Flow chart (*left*) and scheme (*right*) of a portable hyphenated needle-trap device, gas chromatograph, mass spectrometer, and electroantennographic detector (NTD-GC-MS-EAD) [23]. During the enrichment step, the volatiles are trapped in the narrow sorbent in the hollow needle and are then desorbed in the GC injector. The volatiles are separated in the GC column and split by makeup gas between MS and mixing tube. Two Pasteur pipettes—one of which contains a filter paper spiked with a standard solution—are inserted into the mixing tube for monitoring the ageing of the antenna by the monitoring valve. See Fig. 8 for an exemplary measurement

experiment proved that *L. botrana* antennae are 100-fold more sensitive than the quadrupole mass-spectrometer in selected ion monitoring mode, as the antenna can respond to 1 pg/μl (*E,Z*)-7,9–dodecadienylacetate (Fig. 4). The disadvantages of this setup are the relatively high costs and the long enrichment time compared to the portable EAG. However, this method comprises a 100-fold lower detection limit as conventional GC-MS measurements and gives the opportunity to compare concentrations of pheromones in different fields and climates [23].

3.2.3 Portable Single-Sensillum Recording

In 1993, Van der Pers and Minks developed a sensitive apparatus to obtain rapid and specific measurements in the field, allowing real pheromone clouds and sudden fluctuations in pheromone plumes to be monitored [45]. The challenge of a portable single-sensillum recording apparatus lies in the in-field preparation, because of the sensillum's miniscule size and the preparation of the tip, which sometimes has to be cut off [29]. A portable device must be small and rugged. Four micromanipulators need to be positioned accurately in one place: two holding

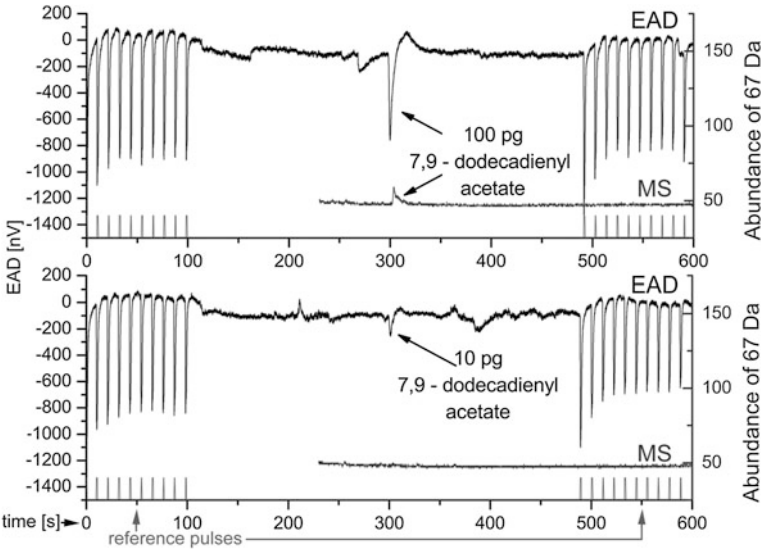

Fig. 8 Comparison of mass spectrometry (MS) and electroantennographic detection (EAD) responses to a pheromone standard eluting from the gas chromatography (GC). The EAD equipped with a *Lobesia botrana* antenna reacts to 1 µl of the pheromone standard (*E,Z*)-7,9-dodecadienyl acetate in acetone at concentrations of 100 pg/µl (*upper chart*) and 10 pg/µl (*lower chart*). The quadrupole MS operated in the most sensitive mode (selected ion monitoring) at m/z 67 (the base peak of 7,9-dodecadienyl acetate) shows a reaction only in the presence of 100 pg/µl in the *upper graph*. The *gray spikes* at the *bottom* of the graphs indicate the opening of the monitoring valve (Fig. 7) for one second each time and result in a peak trace to allow normalization for antennal aging [23]

micro knives to cut off the sensillum tip, one to hold the antenna and the reference electrode, and one for the measurement electrode. A mixing-tube must be positioned at the end at the same spot to apply reference air puffs for the control of the preparation. A microthermistor air speed controller must be placed as near to the probe as possible [45]. Finally, the antenna inside the measurement device must be observable with a microscope, allowing the sensillum connection to be completed. Van der Pers and Minks used *Aegeriamyo paeformis,* whose sensilla are easy to manipulate, although the practical use of portable SSR systems is far from realization [46]. The preparation of a delicate single-sensillum measurement is time consuming and depends on a degree of luck. Therefore, portable single-sensillum devices are currently suitable only in wind tunnels [46].

3.2.4 Miniaturized EAG Systems

Simpler, smaller, and lighter insect-based biological sensors can be developed by combining insect antennae with field-effect transistors (FETs) to produce miniscule measuring devices known as BioFETs. Schöning et al. [47] developed a chip

in which whole beetles or their dissected antennae could be used as sensors. Miniaturized transducers were utilized to convert electrochemical impulses into readable electronic signals on a single chip. All filtering and amplification was accomplished on the same chip, so the system is less sensitive to electromagnetic fields because the electronic conducting paths, which respond to these interferences, are as small as possible. It was possible to obtain dose–response curves with the Colorado potato beetle (*Leptinotarsa decemlineata*) towards in the presence of *Z*-3-hexen-1-ol [47]. Because this assembly is a miniaturized EAG, it suffers from the same problems that occur in large devices, such as responses to nontarget volatiles. Furthermore, it is not easy to adapt the system to different insect morphologies without rebuilding the device because chip size, amplifier, or filter values may need to be readjusted.

4 Behavior-Based Biosensors

Each of the measurement concepts described previously is sensitive, fragile, and requires specially-trained personnel to handle insects or dissect antennae, thus reducing its widespread use. To address these issues whose devices have been developed that are based on the sensitivity and selectivity of the insects whose behavior that can be conditioned to a stimulus. Trained insects can also be collected and loaded into a holder [48], training apparatus [49], and measurement device [50] without the assistance of an entomologist. Living insects can distinguish among reactions from single olfactory receptor neurons because the olfactory nerves are directly connected to the antennal lobe. This overcomes all of the problems associated with measuring the sum of electrical impulses using whole insects or dissected parts in EAG measurements. The insects can also be released after the experiment.

Classical reward-based first-order conditioning (as defined by Carew and Sahley [51]) has been described in several insect species, including the honeybee *Apis mellifera* [52], the fruit fly *Drosophila melanogaster* [53, 54], the tobacco budworm *Heliothis virescens* [55] and its parasite the braconid wasp *Microplitis croceipes* [56], the African cotton leafworm *Spodoptera littoralis* [57], the American cockroach *Periplaneta americana* [58], and the seven-spotted ladybird *Coccinella septempunctata* [59]. Target substances can be associated with measurable behavior. Initially, an entomologist is needed to show that an insect species can explicitly sense a target volatile using EAG, GC-EAD, and behavioral tests. It is advantageous for conditioning if the volatile alone triggers no behavior in nature. The trainable behavior of the honeybee *Apis mellifera* is the proboscis extension reflex [60], which can also be exploited in other insect species. Honeybees extend their proboscis in the presence of sugar; this can be used as unconditioned stimulus (Fig. 9a). The perceivable volatile (conditioned stimulus) is then puffed onto the antenna shortly before exposure to sugar (Fig. 9b). After training, honeybees can reliably remember a conditioned stimulus for at least 48 h

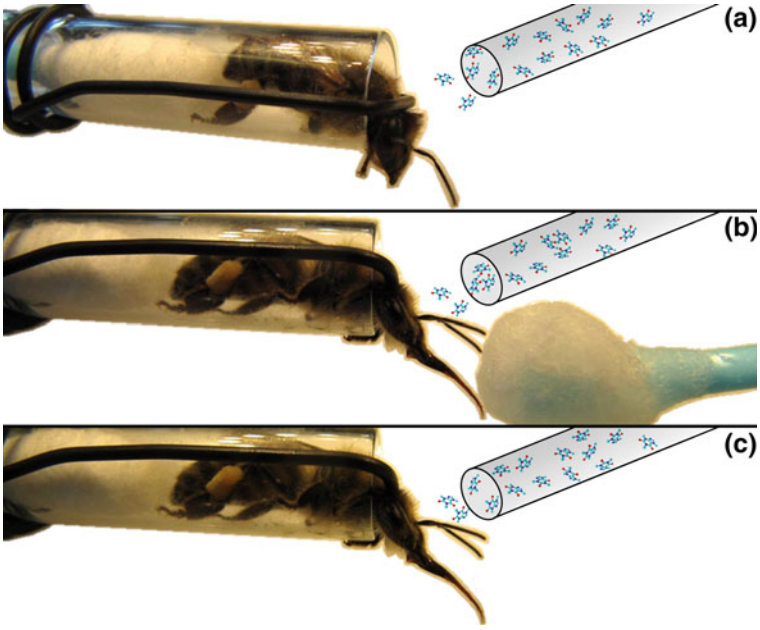

Fig. 9 Conditioning of the proboscis extension reflex in the honeybee *Apis mellifera.* **a** A captured bee in a glass holder with no reaction to the target volatile. **b** The conditioning process involving the presentation of sugar solution as an unconditioned stimulus and a target volatile as a conditioned stimulus. **c** Reaction to the conditioned stimulus without sugar reward

(Fig. 9c), with 80 % of the conditioned response retained even after only three training steps and no subsequent reward [61].

In a bee sniffing measurement setup, which resembles a handheld vacuum cleaner, trained and untrained bees are exposed at the same time to an airstream [50]. If all bees show the proboscis extension reflex, the volatile has a natural origin that would lead the bee to a natural nectar source, such as a flower. If only the trained bees respond, the trained volatile is present in the airstream. Parasitic wasps have been used to detect conditioned stimuli in a setup called "wasp hound" [62]. Here, the measurement setup is even simpler. The wasps can be trained to react to a specific odor by walking to the odor source. The measurement device consists of a small cylinder containing the wasps, with a webcam and a sucking fan on the top and a small hole in the bottom, covered by a sieve to prevent a wasp escape. If no odor is present, the wasps walk freely in the cylinder. If a target volatile is present, the wasps aggregate in front of the opening, which results in a darker measurement chamber. The brightness of the chamber or the position of the wasps can be tracked by the webcam and recorded by a computer [63].

Both apparatuses are inexpensive compared to sniffer dogs and analytical devices [64]. They are particularly useful in cases that are too dangerous for mammals, such as scenarios with toxic chemicals, unstable structures, or explosives [65]. The selected insects must be easy to rear and the training should be

automatable. One drawback is that this detection method can be inaccurate if the target volatiles are masked by a natural unconditioned stimulus.

5 Biosensors based on Insect Odorant Receptors

The drawbacks of the systems described previously can be avoided by using insect ORs and related proteins directly on a sensor layer. The first step is the "deorphaning" of insect odorant-reception genes by identifying particular receptor-ligand pairs [1]. Much progress has been made with vertebrates, but deorphaning in invertebrates is still in its infancy [1]. When a receptor gene is identified, the second step is to express the receptor in modified cell cultures (e.g. bacteria [66], yeast [67], insect cells [68] or *Xenopus* oocytes [69]). Finally, the protein—ligand interaction must be connected to read out using signal transducing techniques based on one of the following concepts [1]:

1. Light-based approaches: measurement of ligand binding by changes in surface plasmon resonance, using luminescence or absorbance.
2. Resonance-based approaches: measurement of ligand binding by changes in the mass that alters the frequency of a constantly-vibrating surface.
3. Approaches based on changes in electrical current or resistance when ligand binding induces a cascade that results in a change of ion concentration.

As discussed previously, the reception of volatiles by insect antennae occurs in a liquid environment, which must be replicated when proteins are used as sensors. The complex events taking place on the membrane of odorant-perceiving dendrites must also be unraveled (Fig. 1g). If the odorant-binding proteins and receptors are used in isolation, the sensor surface can be reactivated by the same ligand molecule over and over again due to the lack of the corresponding odorant-degrading enzyme. These challenges must be addressed in the future before reliable portable systems based on insect odorant receptors can be developed.

6 Gas Sensor-Based Biomimetic Approaches

In contrast to the genetic approaches described above, biomimetic sensors are based on the knowledge of particular volatiles perceived by insects. These volatiles are highly specific for certain events to prevent false-positive signals and energy-wasting behavior (e.g. males following an irrelevant volatile plume, reducing their mating opportunities). The knowledge of specific volatiles perceived by insects can therefore be used to develop sensors for specialized applications; for example, fire detection devices for a sensor grid that monitors the volatiles released by a forest in summer require knowledge of which volatiles from burning wood are unique indicators of forest fires to distinguish false signals by

cigarettes or barbecues. Ciccioli et al. [70] reported more than 200 diverse volatiles emitted from burning pine wood, including alkanes, alkenes, cycloalkanes, cycloalkenes, dienes, trienes, monocyclic and bicyclic arenes, isoprenoids, halogen-containing compounds, alcohols, aldehydes, furans, pyrans, acid esters, and phenols. Some of these compounds are not specific to forests, but Schütz et al. [71] reported that antenna from the beetle *Melanophila acuminata,* which lays eggs on freshly-burned *Pinus sylvestris* trees, can sense six of the reported volatiles, namely α-pinene, carene, 2-methoxy-phenol (guaiacol), and 2-methoxy-4-methyl-phenol (creosol), as well as two additional volatiles not reported by Ciccioli et al. [70], namely 4-acetyl-guaiacol (apocynin) and vanillin. Therefore, fire detection devices for woods with a high density of pine trees should be tuned to those substances. Several gas sensors with different sensing parameters can be used to develop an array of semi-conductive gas sensors adapted to detect a unique profile for each substance [72, 73].

Gas sensors can be used to establish a robust system for other biological applications. For example, the difficult task of quantifying pheromone levels in cultivation areas protected by mating-disruption strategies could be achieved using a sensor array [74, 75]. Pattern recognition and training of the sensor array is necessary, and such robust devices are not yet sensitive or selective enough for field use.

7 Conclusion/Outlook

The utilization of insect olfactory reception in the field is already possible in various ways. In particular, portable EAG and GC-EAD devices allow the measurement of volatiles *in situ*. Instruments based on insect behavior can also be used to measure low concentrations of volatiles, but none of these approaches is suitable for all applications. Measurement speed, accuracy, and sensitivity differ from method to method, and robust field systems are not yet available. Therefore, artificial sensor systems are under development. Most of the systems described in this chapter are used to unravel the secrets of insect odorant reception, including cell-based approaches that yield hundreds of publications each year. The versatility and low cost of trained-insect devices will enhance their commercial development.

The accumulation of data about the insect brain, including the neuronal connectivity in the antennal lobe, will lead to new approaches that exploit insect perception. Minegishi et al. [76] developed an insect–machine hybrid in which the head of a silkworm moth *Bombyx mori* male is connected to an interface that steers a small car-like robot via its motor neurons towards a pheromone source. Kanzaki et al. [77] developed a computer model of insect antennal lobe connectivity that mimics pheromone source-finding behavior; they tested their model on a small robot equipped with two *B. mori* antennae as sensors, allowing them to locate a pheromone source in a wind tunnel. Greater understanding of the neuronal

connections that regulate insect behavior and learning will facilitate powerful pattern recognition models that will enhance multisensory devices.

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Y-Linked Markers for Improved Population Control of the Tephritid Fruit Fly Pest, *Anastrepha suspensa*

Marc F. Schetelig and Alfred M. Handler

Abstract Insect pest control programs incorporating the sterile insect technique (SIT) rely on the mass production and release of sterilized insects to reduce the wild-type population through infertile matings. Most effective programs release only males to avoid any crop damage caused by female fruit flies or transmission of disease by female mosquitoes. Therefore, the females have to be eliminated, preferably in an early developmental stage, during mass rearing. Different systems and techniques have been created for the sex separation of a few insect species. One of these is the transgenic sex-specific fluorescent protein marking of the insects with automated fluorescent-based sorting of the individuals to achieve sex separation. Here we describe the Y-linked integration of fluorescent markers driven by the widely active *Drosophila melanogaster polyubiquitin* promoter in the Caribfly, *Anastrepha suspensa*. Four strains with Y-linked integrations were established with one line expressing the DsRed fluorescent protein marker during embryogenesis. This line now has the possibility for use with automated sex separation in rearing, and the same transgene markers could be used in other insects for similar applications.

Keywords Sexing · Insect pest management · Y chromosome

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Contents

1 Introduction

Integrated pest management (IPM) programs have been used to efficiently control pest species around the world. IPM is a sustainable approach to manage pests by combining biological, cultural, physical, and chemical tools in a way that minimizes economic, health, and environmental risks [1]. An important element of many IPM programs is the sterile insect technique (SIT), which is an environmentally friendly and species-specific program for efficient population control. SIT is an area-wide applied process in which reproductively sterile males are released at overflooding ratios into a wild population of the same species, resulting in preferential mating with wild females in the field that are rendered nonreproductive [2]. For the most effective SIT programs, the species must be mass-reared, sexed early in development (separation of males and females), marked for monitoring, and sterilized by irradiation before release into affected areas. In particular, the production of a male-only population is highly important for large-scale SIT programs because this is most efficient and cost-effective for fruit fly programs [3, 4] and a prerequisite for mosquito programs where adult females are vectors of disease [5, 6]. For most biologically based control release programs, it is highly desirable to have females eliminated early in development to avoid female larval feeding in the mass rearing process [7]. The most commonly used methods are physical, genetic, and transgenic techniques for sex separation.

Physical sexing includes manual sorting using external morphological differences or automated machine sorting based on sex-specific size or color variation. Manual sorting can be labor intensive, but it has been used for sex separation of the tsetse fly, *Glossina austeni*. New knowledge of timing differences between male and female adult emergence has improved sexing and eliminated the need for laborious hand sorting [8, 9]. In the melon fly, *Bactrocera cucurbitae* [10], the mexfly, *Anastrepha ludens* (J.S. Meza, personal comm.), and the medfly, *Ceratitis capitata* [11], strains with sex-specifically colored pupae have been developed. For the melon fly strain, pupae were sexed with high-speed photoelectric sorting machines [12], which could be applied to the other species as well. All physical

sorting techniques have the disadvantage that both sexes must be reared at least through larval stages (and typically to the pupal stage), which increases production costs in mass rearing.

Another option for sexing is the creation of **genetic** sexing strains (GSS) by classical genetic manipulations. In the Mediterranean fruit fly, a GSS has been developed and refined throughout the last 20 years. It is based on two separate components: (i) a *temperature-sensitive lethal* (*tsl*) mutation that is maintained in both sexes as homozygous alleles, and (ii) a Y chromosome translocation that carries the wild-type allele (tsl^+) for the mutation in only the males. In this way, GSS mutant females are eliminated early in development at elevated temperatures, while males survive owing to the Y-linked presence of the wild-type allele [7]. The medfly GSS is currently used in mass rearing to produce up to 4 billion flies per week. The difficulty of transferring such a system to other insects is due to the unpredictable process of isolating *tsl* mutations and the induction of translocations and stabilizing inversions in species that are not genetically well-characterized [7].

In mosquitoes, similar GSSs have been developed since the 1970s based on dominant temperature sensitive (DTS) mutations [13] or insecticide resistance to dieldrin [5, 6]. As with the *tsl* GSS, the mutations could be homozygous in both sexes, while only males carry the rescuing wild-type (WT) allele on the Y chromosome through an induced translocation. However, for dieldrin-dependent GSSs, relatively high semisterility of the males has been problematic for expanding the production capacity, and the waste management of dieldrin-containing solutions and diets in large-scale production is of concern.

Transgenic sexing systems based on lethality systems that are conditionally-repressed by tetracycline were first developed and tested in *D. melanogaster* [14, 15] and then transferred to medfly and *Bactrocera oleae* [16, 17]. These systems are able to kill a high percentage of females when the lethality system is combined with alternative, sex-specific splicing of the medfly *transformer* intron. However, for these systems, the majority of the female lethality occurs at late larval or early pupal stages, which increases mass rearing costs due to the feeding of female larvae. Recently, transgenic embryonic sexing strains (TESSs) were developed in *Anastrepha suspensa* and *C. capitata* [18, 19]. Both systems are also based on a tetracycline repression system, but they induce lethality during embryogenesis by the use of embryo-specific promoters and proapoptotic lethal effectors. For both species, several TESSs were generated with 100 % early lethality as confirmed in large-scale tests [19].

Another transgenic technology, which could be transferred to other insects, is male-specific marking by fluorescent proteins. Such strains have been developed in mosquitoes and fruit flies by inserting a transgene carrying a fluorescent protein under the control of a testis-specific or constitutive promoters [20–23]. Automated fluorescence sorters (COPAS, Union Biometrica) could then be used to separate male and female larvae. In mosquitoes, this approach was successfully used to create a male-only population for *Anopheles gambiae* [24]. Nevertheless, female larval rearing is still necessary; for fruit flies, late larval stages may be too large for the automated sorting machine. Fluorescent marking of physically smaller

embryos or early larval stages would improve the throughput of the separation system and avoid larval feeding during mass production. To address these limitations, we describe the creation of male-specifically expressed Y-linked transgene integrations in *A. suspensa* and the evaluation of early male-specific fluorescence for separating males and females.

2 Results

2.1 Random Integration of Transgenes in A. suspensa

To generate male-specific fluorescent marked strains, the markers need either to be linked to the Y chromosome or be under the influence of a male-specific promoter/enhancer. Because there is no site-specific targeting available for *A. suspensa* to insert markers directly onto the Y chromosome or other preferred male-specific chromosomal loci, three *piggyBac* vectors (423_attP_PUbEGFP, 437_attP_PUb-DsRed, and 443_attP_PUbEGFP) were integrated into the fly genome by germline transformation. The vectors were integrated into an *A. suspensa* WT strain, establishing 5 to 10 independent lines each by screening for epifluorescence of the PUb-EGFP or PUb-DsRed.T3 marker. Randomly, 4 out of 20 independent lines expressed the fluorescent markers male-specifically (437_M5A, 437_M7A, 423_M10B, 443_M7m5; Fig. 1). Transgenic males from these lines were backcrossed to WT females to determine Y chromosome transgene linkage of their offspring by epifluorescence screening. All male progeny from the strains 437_M5A, 437_M7A, 423_M10B, and 443_M7m5 expressed the respective fluorescent marker, whereas none of the females exhibited any marker expression. This backcross was repeated with the selected males crossed to WT females and resulted again in 100 % fluorescently marked males. This indicated Y-linkage of the fluorescent marker because an autosomal insertion would not yield expression in all males.

2.2 Molecular Characterization

For each transgene, the integration site flanking sequences were isolated by thermal asymmetric interlaced (TAIL) polymerase chain reaction (PCR), confirming that all were canonical *piggyBac* integrations into genomic TTAA sites. For 437_M5A, the genomic flanking sequences were identified as a microsatellite locus similar to a previously described *A. suspensa* 1-5E microsatellite clone by the BLASTN algorithm using the nr database at NCBI [25], whereas the integration site of 443_M7m5 had similarities to an *A. suspensa mariner* transposase pseudogene (accession number U04466). Interestingly, two of the four integrations, 437_M7A and 423_M10B, occurred independently in the same intron of a

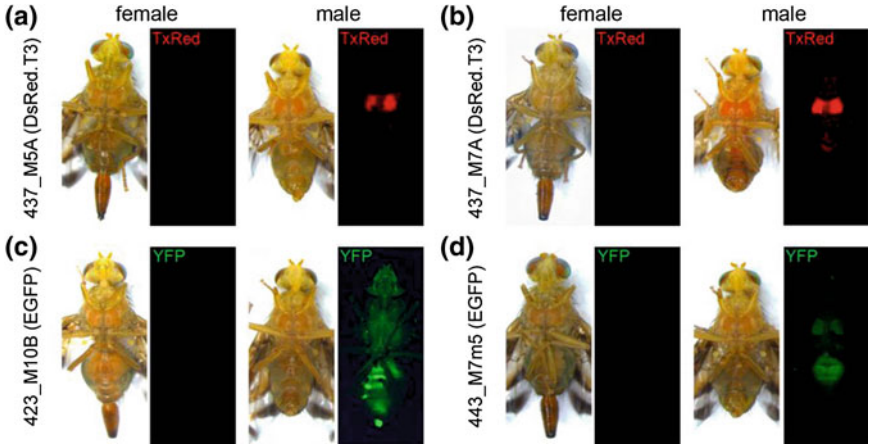

Fig. 1 Male-specifically marked *A. suspensa* strains. All lines show male-specific expression of the PUbDsRed.T3 (**a** and **b**) or the PUbnlsEGFP marker (**c** and **d**). Males and females from each line were observed under brightfield conditions (*left panel*) and epifluorescence microscopy with the respective filter sets, YFP or TxRed (*right panel*)

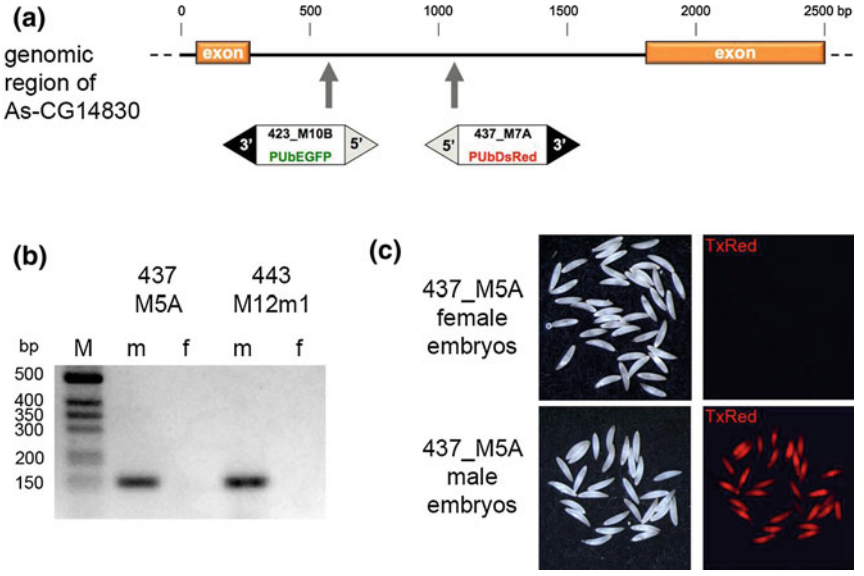

Fig. 2 Genomic verification of integrations and embryonic fluorescence. **a** Genomic structure of *As*-CG14830 (*A. suspensa* cognate of *D. melanogaster* CG14830) indicating independent *piggyBac* integration events in the lines 423_M10B and 437_M7A. Base pair numbers indicate the isolated region of the *As*-CG14830 gene (see also Supplementary data). **b** PCR on male (*m*) and female (*f*) genomic DNA of 437_M5A and 443_M12m1 targeting the *tTA* and *EGFP* gene, respectively. **c** Embryos from 437_M5A males and females under brightfield conditions (*left panel*) and epifluorescence microscopy with the TxRed filter set (*right panel*)

gene homologous to the male-specifically expressed gene, CG14830, in *D. melanogaster* [26] and a testes developmental protein, *nyd-sp29*, in *Ae. aegypti* (Fig. 2a). The Y-linked integration of the transgene in the strains 437_M5A and 443_M7m5 was further verified by PCR to the isolated flanking regions to female and male genomic DNA (Fig. 2b).

2.3 Sex Separation of Fluorescent Embryos

To perform automated embryonic sorting and avoid larval rearing, detection of embryonic fluorescence is essential. Therefore, embryos and larvae from all Y-linked strains were examined for the earliest visible fluorescence. In 437_M7A, 423_M10B, and 443_M7m5, expression of DsRed or EGFP was not detected until the third instar larval stage. The only strain expressing the DsRed by late embryogenesis (55–64 h after egg laying) and the first larval instar was 437_M5A. A total of 200 embryos from this strain that did (97) or did not (103) express DsRed were then manually selected and maintained on two different larval diet plates. From the DsRed-expressing embryos, 69 survived as male-only adults, whereas 72 adult females enclosed from the nonexpressing embryos. This demonstrated that progeny from the line 437_M5A could be separated during embryogenesis to create a male-only population (Fig. 2c).

3 Discussion

Here we describe the generation of the first Y-linked markers for use in embryonic sex separation in the tephritid pest, *A. suspensa*. Four transgenic strains were established with one expressing a fluorescent protein during embryogenesis. Integrations in *A. suspensa* with male-specific expression patterns thus occurred in 20 % of independent strains generated in this experiment, although this relatively high frequency is likely to differ for other insects and with the use of other transgene vectors. The three different constructs tested in *A. suspensa* were distinct except for *piggyBac* vector sequences and a 220 bp *attP* landing site, so it is unlikely that transgene vector structure was responsible for the generation of the male-specifically expressing lines. Interestingly, two vectors integrated independently into the same intron of a gene having homology to a testes developmental protein gene in *Ae. aegypti,* although testis-specific fluorescence was not detected. At present, it remains to be determined whether male-specific enhancers effect the sex-specific expression of the fluorescent marker.

It is more likely that Y-linkage for the transgene is primarily responsible for the male-specific marker expression. Although a molecular determination for Y-linkage has yet to be determined for either integration (due to a lack of Y-specific sequence data), Y insertions are supported by backcrosses of fluorescent-marked males to WT females that resulted in only fluorescent male progeny.

Thus, for the first time, a Y-linked embryonic fluorescent expression line, 437_M5A, has been created that can be used to separate male from female embryos to generate a male-only population. In addition, the #437 transgene has an *attP* landing site that can be used to integrate new transgenes specifically on the Y chromosome, providing a reliable mechanism for future male-specific modification of these strains.

The marking of males by fluorescent proteins had been previously achieved in several other insects [20, 22, 27], but fluorescence did not appear before late larval stages, with the exception of one system for the Mediterranean fruit fly [21]. Because feeding larvae increases production costs in large-scale rearing, developing embryonic sexing systems remained a high priority. The strategy of randomly integrating markers driven by the *polyubiquitin* (*PUb*) promoter onto the Y chromosome is an alternative to physical, genetic, or other transgenic sexing techniques. Such markers have the advantage that they can be easily transferred to new species, whereas other highly efficient embryonic transgenic sexing systems need more adaptation to the host species [18, 19]. Development of Y-linked markers can be typically achieved in a shorter timeframe than generating classical genetic sexing strains [7], but a limiting factor is the need for separation by automated fluorescence sorting machines. Currently, these machines can sort material only up to a certain size, which can be problematic for insects having large embryos or first instar larvae. Recently, sorting fluorescently labeled larvae of *An. gambiae* by the automated COPAS system has been evaluated [24]. This was highly accurate for both transgene heterozygotes and homozygotes; however, a high-throughput evaluation over several days has yet to be conducted. Presently, the calculated, sortable numbers are not sufficient for SIT programs using 100 millions of males weekly. In the future, further improvements on sorting machines could eliminate this bottleneck, allowing high-throughput separation to be possible for many insect species.

4 Materials and Methods

Insect rearing. An inbred wild-type colony of *Anastrepha suspensa* (Homestead, Florida) was maintained at 25 °C and reared under standard laboratory conditions [28, 29]. All embryonic, larval, and pupal stages of *A. suspensa* were reared at 27 °C and 60 % humidity on a 12 h light:12 h dark cycle.

Cloning. The vector #423 (pXLII_PUbEGFP_f_attP235_SV40-slamA-AstraIntron-slam_hs43-TRE) was created by ligating a *Fse*I/*Asc*I cut 5.6 kb *attP235_SV40-slamA-AstraI-slam_hs43-TRE* fragment into *Fse*I/*Asc*I cut vector #1419 [30]. The vector #437 (pXLII_attP_PUbDsRedT3_Ccvas-tTA) was generated by ligating the *Asc*I fragment *Ccvas-tTA* from M493 to the *Asc*I cut vector #1425 [30]. To create M493, the *Ccvas* promoter was *Sma*I/*Xba*I cut from M429 and ligated into #1215 [31].

The vector 443 (*pBXLII_PUbEGFP_TREhs43-CctraI-Alhid^{Ala2}_loxN-3xP3-FRT-AmCyan_lox2272_loxP_attP235*) was described previously [19].

Germline transformation. Germline transformation experiments were performed by microinjection of the *piggyBac* constructs #423 or #437 (500 ng/μl) together with the *phsp-pBac* transposase helper plasmid (200 ng/μl) into WT *A. suspensa* embryos as described [32]. G1 offspring were selected by EGFP or DsRed epifluorescence using a Leica MZ FLIII microscope and the YFP (ex: 500/20; em: 535/30) or TxRed (ex: 560/40; em: 610 LP) filter sets.

Independent homozygous strains were established by single pair inbreeding for successive generations with testing by segregation analysis of transformants outcrossed to WT flies. Transgenic *A. suspensa* lines carrying the #443 *piggyBac* cassette were generated and described earlier as a lethal effector construct [19].

Isolation of transgene integration flanking site sequences. Flanking sequences of #423, #437, and #443 transgene integrations were isolated by TAIL PCR or inverse PCR. TAIL PCR conditions were as described previously [33]. Oligos used for the isolation of the 5′ *piggyBac* vector insertion-site flanking sequences (of 423_M10B, 437_M5A, and 443_M7m5) by TAIL PCR were the degenerate primer AD3 (AGWGNAGWANCAWAGG) and the specific primers L1_P882 (CATTTTGACTCACGCGGTCGTTATAGTTC), L2_P883 (CAGTGACACTTACCGCATTGACAAGCA), and L3_P884 (CGACTGAGATGTCCTAAATGCACAG). Oligos for the 3′ *piggyBac* flanking sequence (of 437_M5A and 443_M7m5) were the degenerate primer AD3 (AGWGNAGWANCAWAGG) and the specific primers R1_P885 (ACCTCGATATACAGACCGATAAAACACATGC), R2_P886 (GTCAATTTTACGCATGATTATCTTTAACGT), and R3_P887 (CGTACGTCACAATATGATTATCTTTCTAGG). The PCR conditions and the generation of DNA pools for inverse PCR are described in Schetelig and Handler (2012a). The 3′ flanking sequences of 423_M10B and 437_M5A were isolated by inverse PCR using *Xho*I-digested genomic DNA and the oligo pairs P144/P830 (CCTCGATATACAGACCGATAAAACAC/CTTTTATCGAATTCCTGCAGC) and P144/P777 (CCTCGATATACAGACCGATAAAACAC/CCGACATGACACAAGGGGTTG), respectively.

Verification of Y-linked integrations. First, the expression of fluorescent markers in adult flies was used to identify male-specifically marked strains by epifluorescence microscopy. To confirm Y-linked transgene insertions, fluorescent males were subsequently backcrossed to WT *A. suspensa* females for two generations and the number of fluorescent/nonfluorescent progeny assessed. Epifluorescence was also used to assess embryos and larvae from male-specifically expressing lines for the earliest possible stage of marker detection using the YFP or TxRed filter sets.

Secondly, transgene integrations in the lines 437_M5A and 443_M7m5 were molecularly verified by PCR of genomic DNA (PCR conditions: 2 min at 95 °C; 30 cycles of 20 s at 94 °C, 30 s at 59 °C, 20 s at 72 °C). The oligo pairs P756/P757 (GCTGCTTAATGAGGTCGGAATCG/TGGTGCCTATCTAACATCTCAATGG), binding to the *tTA* gene of the transgene #437, and P913/P914 (CAGAACACCCCCATCGGCGACGGC/TACTTGTACAGCTCGTCCATG), binding to the EGFP marker of #443, were then used on male and female genomic DNA.

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5 Supplementary data

Sequence of the A. suspensa CG14830 homolog. TTAA piggyBac integration sites of the plasmids 423 (green) and 437 (red) are indicated. Possible exons predicted by comparison to D. melanogaster CG14830 are marked (orange).

CGAGACGACGCTGCCTGCTATGATGATATTCTGGGCGGAGGGATAATAACGGCCATATCGGAGCATCGACC
TTCAGATCCTGAAAAGTTGCTGGGAATTGGTGATTATAACAGCAGTTTCCGCATGATGAAATTACCTAGCA
GTTTCTTTACGGCTGAGCCTAAAGAATTAGAGGTTCGTTCACATTCTTCAAAATTTTGATATATATATATA
TATATATACATATATTTATTTAATTGTTGGAAGACATGGCCGGCGAGGACAATCGCAACCTACTTCGGGTA
TGTAGAATCGTACTACACCATTAGTTTTTTCAGTTTTCAGCACGCATTCATAAAAACTTTTGTTTGAAAAT
TTTGCCTTCACCGTTCACAGTTTTCGGATCACAAGTCTTTAGAACTTGCTATTTTCAATCTTATACTTCCA

423_M10B integration

CAAAAATGTTGCTCAAATATTAGATGTTGCTCTGAACATCAAAATTACAAGCCCTAAATGTTG**TTAA**AGCT
GAAATTGGGGTTTTTATACATAATTGCAGCATTTCGTTCATTTTGCTACGCATGGGACATATAGTATATAC
GAGTTCGCAATGTCATACGTCGACATGCCGTTAAAATTAAATTTTTTGTATCACCATAAGAATCGTTTTGG
CCTTCACACTCCCACGGGTTCAGCCGAACACGATGGAATAATTCACCAGATTGCCACTCCTTTAAAGCATT
AGGATTCTGGATATAAACTTCATTCAATGTTCGCTGATTTATGCTGGAATTTTATTGTCGAAAATTAGCCT
TTTTTAAAGCTTTTTCCGCCGATGTTACAATTAAAATACTTCTGCTTTGGGATTTTGCACCCAGAATATGA
TTCTGAATATCTTAGCTAGAGGAATTTTGTCGAAAGACCAGATGAAAGTGAAGAAAAAAGGCTAAAACACC
TCAAAAATGTTCCTTGGAGGCAAAATTTTCCAAAAAATGTTTTATGAAGACGAGCGGAAAAACTGGTGTTG
CAGTTCATTTTCCTATACCGAGAATAGGATAAAACCTTCCTTTCCGCGCATGGCTTTTCTTGTGGTTATAA
CAGTGTAATAAGAGATTCTCTAAGTTTTGCATAATGGCAGAATAGCAAAAGTATAAGAGCATAGAATTGTA

437_M7A integration

CCAACC**TTAA**AATAAACAACGACATTTACATGAGTATGCTGTAGCTATTCTGGCAGTTATTGGATATTTTG
TGTGTGTTTTTTGTTATGTCATTGCAAGTATTAGAAAACTTTGCAATTCTTGCACCTTGCACAGGTTTTGC
AAGACATAGAGAATCCTAATTCGTTGAATATTTATTACAGCATTACAGATAAGATCATATTTTCTGATGAA
TTACTGTGCAAAAATCCGTGTAAATCAAATCCCTGAGCGACCTTCGTCAGACGAAAATGTGCTGTCAAAAT
AAAAACACACCCATATACAAATATGAGATTACATATGATATTGACACAACCAAAGAGAAGCGTTAGACAAA
CTTCGTCGATAACAGAACTATTTCTAATTTTCCCCTTGCCCCCGGCCGTCAAAACCGTTGAAAATGTAAAC
AAATCCTTTTCACAAACGTTGCCTCACTTGCAGAAATGCCNTGCACATTATGTATAAACAAGAAATTTAAA
ACTTTGATAATTAGAATTAACCGAAAAATTTTGAGTTCATCTTTTCACTTCTCATGCGAATTTCGTTTTGG
CTCTATTTTACTGTGCAAAAATTTCAATCGACGGTATCGCTTCGCCCGCCGAAGTTCGCTTGTCGATTTGA
TTCTGTTTATACTATACTACTATGCTTATTTTTCATCCTAGAGAGTTAAAGAGTATTTTAGTAGAGAAGAA
AATCGCAAAAAATCTATTCAAGCATGGGAACAACAGTTCTTCGAAAATAATCGTGATATAATTGAAGCAAA
ACGTCAGGCCGAATCCGATGCCAGGAAAGAGCTTGAACGGCTTGAGAAGGAGTATTCACTAAATGCCACAC
GTAAAGTCAAAGATGAAGCTGGTGATAAAAGGTATATAGTAAATGTTCGAAAAAAGTGTTTTTTTATAAAC
TGAAAACATTTTTTATTGCATATTTCCTTAAGGAATGAACACAAAAATCTTTCATACACAGAACGTATAAA
ACATAAATGGGATGAATTGAATTTGAATCGCCAGCTAACTATTTTAATGTCTCGTAAACGTGTTGATGAAG
AAAAACTAGAGCGAGAAACTAGACTTGAAAAATATCATTTGTCATATGAGGCAGCAAAGAAACAGTCATTA
ATTCGCATCCAGCAGCGTGTGGGAGAAGAAGTAGCGGCTGTAAGGGCAAGGATACTACCACATGAAAACGT
CGATCTTCAACGCATCGATATGATACTTTCATCTGTACAAGTGTTAATGGAGACAGTTGATGATTATAATG
ATATTGAGATGGAGTCCAATGAAGTGGTGAACAATTTTGATGCCTTTGCTACTACACT

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Transgenic Approaches to Western Corn Rootworm Control

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Abstract The western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) is a significant corn pest throughout the United States corn belt. Rootworm larvae feed on corn roots causing yield losses and control expenditures that are estimated to exceed US$1 billion annually. Traditional management practices to control rootworms such as chemical insecticides or crop rotation have suffered reduced effectiveness due to the development of physiological and behavioral resistance. Transgenic maize expressing insecticidal proteins are very successful in protecting against rootworm damage and preserving corn yield potential. However, the high rate of grower adoption and early reliance on hybrids expressing a single mode of action and low-dose traits threatens the durability of commercialized transgenic rootworm technology for rootworm control. A summary of current transgenic approaches for rootworm control and the corresponding insect resistance management practices is included. An overview of potential new modes of action based on insecticidal proteins, and especially RNAi targeting mRNA coding for essential insect proteins is provided.

Keywords Cry toxin · *Diabrotica* · Refuge · Resistance management · RNAi · Thuringiensis · Transgenic maize · Western corn rootworm

Abbreviations

BBMV	Brush border membrane vesicles
Bt	*Bacillus thuringiensis*
CERA	Center for Environmental Risk Assessment
EPA	Environmental Protection Agency
IRM	Insect resistance management
WCR	Western corn rootworm

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1 Introduction

1.1 History of Western Corn Rootworm Management Practices

The western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), is arguably the single most important pest of field corn, *Zea mays* L. [1–3], throughout most of the US corn belt both in terms of crop losses and the use of synthetic insecticides. WCR is a univoltine pest that overwinters in the egg stage [4]. Larvae hatch in late spring to early summer, depending on geographic location and annual weather conditions. Larvae have an obligatory relationship with grasses (Graminae), especially corn, and obtain nourishment and cause the majority of economic damage to corn via root feeding as larvae. A recent economic analysis estimates that costs of control and yield loss associated with WCR damage exceed US$1 billion annually [1]. Historically, crop rotation to a non-host crop, such as soybean, *Glycine max* (L.), and planting time or postemergence insecticide application in continuous corn have been the primary control methods used against corn rootworms.

The history of WCR as a corn pest has been referred to as “one of remarkable adaptability and invasiveness” [2]. Managing corn rootworm populations to minimize economic loss is extremely difficult, because of its remarkable capacity to evolve resistance to both chemical insecticides [2, 5–9] and cultural control practices such as crop rotation [2, 10]. In addition to being highly adaptable, WCR is a formidable invasive pest. In the second half of the twentieth century, WCR underwent a dramatic range expansion out of the Great Plains, eventually reaching

the East Coast [9, 11]. More recently, it has invaded a significant portion of central and southeastern Europe along with numerous disconnected outbreaks in western Europe [3, 12].

1.2 Invasiveness

The ancestral origin of WCR and the underlying cause of its expansion across the US corn belt remain somewhat speculative. It was first described as a maize pest in Colorado in 1909 [13], and prior to the mid-twentieth century, population densities were low and reports of its presence were scattered and uncommon [9]. Native prairie grasses, which may be the ancestral host of WCR, are likely to have supported low populations prior to widespread corn plantings [14, 15]. By the mid-1940s, both WCR and severely damaged corn had become common in central Nebraska, marking the beginning of its dramatic eastward range expansion. By the mid-1980s, expanding populations of WCR had reached the Atlantic coast from New York to Virginia [16, 17]. The initiation of the range expansion coincides with an increase in continuous corn production that became a common practice after World War II and that was facilitated by the development of irrigation and the introduction of synthetic fertilizers [2, 9]. Continuous corn production without rotation to other crops is believed to be necessary for WCR densities to increase because of the insect's fidelity to corn as a larval host.

Expansion of WCR's range did not stop when it reached the Atlantic Ocean. In 1992, a WCR infestation was detected within a small field of corn near the Belgrade airport [18]. Since its initial detection near Belgrade in 1992 [18], WCR has become established throughout central and southeastern Europe along with numerous disconnected outbreaks in western Europe [12, 19]. A precise source of these invasions is still uncertain; however, Miller et al. [19] concluded that there had been at least three direct introductions into Europe from North America and that there have been additional intra-European introductions from central and southeastern Europe. More recently, Ciosi et al. [20] utilized microsatellite genotyping to identify as many as five independent introduction events from the northern United States into Europe and suggested multiple invasion events highlighting the potential for additional introductions.

1.3 Adaptation

There are numerous examples of WCR adaptation to uniform, large-scale pest management practices including both chemical and cultural control. Prior to the mid-1990s, crop rotation between corn and non-host crops, such as soybean, in much of the eastern corn belt had eliminated the need for rootworm-targeted insecticide applications to first-year corn. However, in areas of Indiana, Illinois,

western Ohio, and southern Wisconsin, a variant of the WCR has circumvented these crop-rotation strategies [10]. Extended egg diapause, which has been documented in the closely related northern corn rootworm, *Diabrotica barberi*, was initially thought to be responsible for first-year corn damage [21]. However, it has been confirmed that this variant disperses from corn fields to feed and oviposit in soybean fields and other non-host crops [22–25], thus increasing the probability that larval progeny from eggs deposited outside corn fields will cause damage to corn planted in those fields in the following year. As a result, both soil and foliar insecticide applications are now being used in an attempt to prevent rootworm damage in first-year corn in these areas. The defining characteristic of the rotation-resistant population of WCR is a broadened ovipositional host range [2]. Tremendous abundance of rootworm adults outside of corn as well as herbivory by adults on soybean foliage are also symptomatic of the problem.

Resistance to chemical insecticides has also been well documented in WCR. Perhaps the most dramatic example of resistance evolution in terms of the affected area and intensity of resistance involves WCR resistance to cyclodiene insecticides. These compounds were commonly used as soil treatments for the control of both western and northern corn rootworms from the late 1940s to early 1960s. Benzene hexachloride [26], aldrin, chlordane [27], and heptachlor [28] were the recommended active ingredients for control of root-feeding larvae during this period. By 1959, almost 1 million kg of aldrin were used as a soil insecticide in Nebraska [29]. Control failures with these compounds were first noted in Nebraska in 1959 [30], and further evaluations in 1960 [31] and 1961 [32] revealed the magnitude and rapid development of the resistance. During 1961, WCR adults were collected from different fields in Nebraska and susceptibility to aldrin and heptachlor was determined by topical application [33, 34]. Differences in susceptibility as high as 1,000-fold were detected among field populations and provided the first direct evidence for resistance evolution in WCR.

After the development of cyclodiene resistance had become widespread, organophosphates and carbamates became commonly used as soil insecticides. In addition, foliar application of these compounds that target adult females to reduce egg laying and economic damage from the subsequent generation [35] had become widely adopted. In some areas of Nebraska, aerially applied microencapsulated methyl-parathion was used almost exclusively [6] over relatively large contiguous areas in consecutive years. Control failures of aerially applied methyl-parathion were first reported in the early 1990s, and resistance to organophosphate and carbamate active ingredients was documented in rootworm adults from a number of Nebraska populations [6]. The distribution of resistant rootworms was initially restricted to areas where adult management had been practiced in excess of 10 years, whereas areas relying on soil insecticides and crop rotation apparently remained susceptible.

These examples highlight not only the remarkable capacity of WCR to evolve resistance to divergent pest management strategies, but also the invasive nature of resistant populations and movement of resistance alleles beyond the areas where resistance was first identified. In the case of cyclodiene resistance, the onset of

resistance coincided with the rapid eastward range expansion. This apparent increased invasiveness has been suggested to have resulted from increased fitness and a change in behavior associated with the resistance [5]. Although the exact causes for WCR range expansion are uncertain, it is clear that the source of the invading populations that became established throughout the US corn belt originated in areas where resistance had become widespread (i.e., Nebraska) and that all populations east of the Missouri River have been and continue to be highly resistant to cyclodiene insecticides. Surprisingly, these high resistance levels have persisted in spite of apparently reduced selective pressures since the cyclodienes used as soil insecticides were banned from use in the United States in 1972. However, considerable variation in resistance levels among populations has been reported previously. Ball [29] reported that a significant decline in resistance was noted between 1962 and 1981 among Nebraska populations of WCR and associated this decline with reduced soil residues of the aldrin metabolite, dieldrin, which possesses similar toxic properties [36]. Parimi et al. [7] reported the presence of high levels of resistance in both laboratory-reared and field-collected adult WCR based on topical bioassays with aldrin. Similar to Ball's results [29], considerable variation in resistance levels was detected among populations with a general decline in resistance among Nebraska populations and consistently higher levels of resistance in more eastern populations.

A recent investigation of cyclodiene susceptibility among invasive European populations has revealed that among nine different field populations examined, all were highly resistant to aldrin [12]. No heterogeneity in aldrin resistance was detected in either of the two independent outbreaks identified in Europe. Given the very low rates of mortality observed in this study, the resistance may be considered fixed in the field samples examined. Aldrin resistance is likely to have been introduced independently, at least twice from North America into Europe as there is no evident selection pressure to account for an increase of frequency of aldrin resistance in each of the invasive outbreaks in Europe [12]. The potential for cyclodiene resistance to be useful as a marker for identifying the area of origin for invading European populations has yet to be examined.

Movement of resistance has also been associated with the behavioral variant that allows resistance to crop rotation. Since its first identification in a single county in Illinois in 1986, the rotational-resistant variant of WCR has spread 10–30 km/year depending on the directions of the prevailing storms and winds and now occupies most of Illinois and Indiana, and has been detected as far away as Ontario, Canada [9]. Similar movements of resistance-conferring alleles have been observed with organophosphate resistance in Nebraska. Initial sampling of rootworm susceptibility in 1996 indicated the presence of two distinct resistance areas based on susceptible populations that separate the two regions [37]. However, by 1998, significantly increased resistance levels were observed in areas previously identified as being susceptible [3, 9] and that resistance was detected in areas where selection intensity is likely to have been minimal because adult management was not practiced. Understanding the spread of resistance and movement of resistance alleles is critical to resistance mitigation efforts and is becoming

increasingly important to regulatory decisions regarding new technologies such as transgenic plants that have mandatory resistance management requirements.

2 Transgenic Maize Technology Based on *Bacillus thuringiensis* Cry Proteins for Western Corn Rootworm Control

2.1 Overview

Insecticidal proteins incorporated as transgenic traits in maize hybrids have proven to be effective for providing tolerance to WCR in the field. To date all commercialized maize lines resistant to WCR damage incorporate traits based on the parasporal, crystalline (Cry) proteins derived from the soil bacterium *Bacillus thuringiensis* (Bt), although other protein classes are being investigated.

Cry toxins comprise a broad range of insecticidal proteins with varying structures and insect specificity [38–41]. Many Cry toxins are comprised of three domains with the following functions: (1) domain I is a bundle of seven alpha helices that are essential for membrane insertion and pore formation; (2) domain II consists of three antiparallel beta sheets, with loops that are involved in receptor binding, and (3) domain III consists of two twisted antiparallel beta sheets arranged in a beta sandwich; domain III is also involved in receptor binding and target insect specificity [38, 42].

In general terms, Cry proteins intoxicate insects by disrupting midgut epithelial tissues following oral ingestion [43]. Cry proteins are often produced as protoxins that are first solubilized in the insect midgut and then proteolytically processed to yield smaller activated polypeptides. The activated Cry proteins then bind to specific receptors on the surface of insect midgut epithelial cells. Receptor binding is followed by assembly of activated Cry proteins into pores that result in colloid osmotic lysis of midgut cells due to an influx of solutes from the midgut lumen. Cell lysis leads to disruption of the midgut epithelium and, ultimately, death of the insect larva.

Cry proteins act on a very specific range of susceptible insects. As a result, Cry protein sequence homology families commonly have a spectrum of insecticidal activity that is orders of magnitude less active to taxa outside the primary specificity range. Because of this specificity, Cry proteins are considered to be an environmentally benign insect control technology relative to traditional neurotoxic insecticides [40, 44].

Transgenic maize events expressing Cry proteins for control of WCR have achieved regulatory approval for cultivation in the United States, Canada, Brazil, and Argentina [45, 46]. Insect resistance traits for WCR were first registered in the United States in 2003 with Monsanto's YieldGard® event MON863 rootworm trait. In 2005 Dow AgroSciences introduced the Herculex RW® DAS-59122-7

event, followed in 2007 by Syngenta Seeds Agrisure® MIR 604 event. Traited Bt technology for control of WCR has achieved rapid adoption in the United States because transgenic maize lines provide superior yield compared to nontransgenic isolines and reduce farmer input costs [47]. The current trend in deployment of Bt technology for corn rootworm control is to combine WCR resistance traits by traditional breeding or molecular stacking to generate corn lines expressing dual modes of action (also known as trait "pyramids"). Corn lines producing insecticidal proteins that target independent receptors within the insect midgut have the potential to protect product durability by delaying the development of resistant insect populations [48]. To date only two major Cry protein classes have been developed for transgenic control of WCR. This situation is stimulating research for new modes of action effective against WCR.

2.2 Cry Proteins in Registered Transgenic Maize Events

2.2.1 Cry3 Toxins

Cry3 toxins are one of only two Cry protein classes registered for transgenic corn products resistant to WCR damage [45]. The Cry3 family of Bt proteins are best known for their insecticidal activity on Coleopteran pests [40]. These proteins are classical three-domain Cry toxins [39, 49, 50]. In certain susceptible insect pests, Cry3Aa1 mode of action involves interactions with protein receptors that include cadherin in *Tenebrio molitor* [51] and metalloprotease in *Leptinotarsa decemlineata* [52]. Consistent with these findings, a cadherin of WCR has been suggested as a possible receptor for Cry3Aa and Cry3Bb [53]. Native Cry3 proteins are relatively ineffective on *Diabrotica* spp. However, modified versions of both Cry3Aa1 and Cry3Bb1 have been developed for effective control of WCR [54–56].

mCry3Aa1

Cry3Aa1 was the first reported Coleopteran-active Bt insecticidal protein [57, 58]. Cry3Aa1 in its native form has been reported as ineffective against *Diabrotica* spp. [57, 59, 60]. To improve Cry3Aa1 activity on WCR, Walters et al. [54] used information on toxin activation to engineer a modified Cry3Aa1 variant designated mCry3A. It was previously known that Cry3Aa1 is processed by insect midgut proteases, or chymotrypsin, in a loop between alpha helix 3 and alpha helix 4 of domain I [61, 62]. A modified Cry3A (mCry3A) was engineered to contain a chymotrypsin/cathespsin G protease site at this location in the protein, resulting in more rapid proteolytic conversion of mCry3A to the activated form of the toxin. As a result, mCry3A exhibited specific binding to WCR midgut brush border membrane vesicles (BBMVs), whereas native mCry3A did not. Together, enhanced toxin cleavage and binding to insect midgut membranes resulted in mCry3A toxicity to WCR larvae that was superior to native Cry3Aa1. Maize event MIR604 expressing mCry3A was registered for cultivation in the United States in 2007 [45].

eCry3.1Ab

Walters et al. [55] again successfully modified Cry3Aa for WCR activity using a second approach based on intermolecular exchange of domain III. The basis for this approach lies in the fact that naturally occurring Cry proteins are known that result from evolutionary exchange of domain III variable regions [63]. The ability to swap domain III regions, or parts of domain III, for improved activity and spectrum had been reported previously [64, 65]. Walters et al. [55] generated a hybrid protein resulting by exchange of the domain III variable region components from a Lepidopteran active toxin, Cry1Ab, with a Cry3A region. The resulting protein, eCry3.1Ab, has higher activity against WCR than Cry3Aa. Another unique attribute of Cry3A.1Ab is that it binds WCR midgut BBMVs at sites independent of those that bind mCry3Aa. The lack of competitive binding between mCry3A and eCry3.1Ab suggests that these proteins might act via independent mechanisms on WCR midguts and are therefore potentially useful in trait pyramids to delay resistance development in WCR populations. Maize event 5307 expressing eCry3.1Ab recently received United States EPA and FDA approvals for use in food and feed (http://www.fda.gov/Food/Biotechnology/Submissions/ ucm304082.htm; http://www.gpo.gov/fdsys/pkg/FR-2012-08-08/html/2012-19319.htm).

Cry3Bb1

Cry3Bb1 is active on WCR and other Coleopteran pests such as the Colorado potato beetle, *Leptinotarsa decemlineata* [66]. Cry3Bb1 is a three-domain Bt protein with structural similarity to many other Cry proteins [50]. Consistent with the conserved protein structural features, Cry3Bb1 has been demonstrated to function by ion channel formation in planar lipid bilayers [67]. Cry3Bb1 was the first Bt protein trait commercialized for corn rootworm control in transgenic maize event MON863 [45]. Cry3Bb1 as expressed in events MON863 and in a newer event MON88017 is a modified protein with 6 amino acid residue changes compared to the native sequence [56]. Grower adoption of WCR-protected corn has been exceptionally rapid, with approximately 50 % of corn acres being planted with corn hybrids containing WCR traits [47]. Widespread use of the Cry3Bb1 trait, has resulted in reports of field-evolved populations of WCR that are resistant to Cry3Bb1 [68, 69].

2.2.2 Cry34Ab1/Cry35Ab1

The second major class of Bt toxins developed for transgenic maize lines protected from WCR injury are the binary Bt crystal proteins Cry34Ab1 and Cry35Ab1 (Cry34Ab1/Cry35Ab1) [45]. Cry34Ab1/Cry35Ab1 function together as oral toxins of WCR larvae [70]. Cry34Ab1/Cry35Ab1 are very different from the Cry3-type proteins described above. Cry34Ab1 is one example of a family of 14 kDa proteins that have no sequence homology beyond the Bt Cry34 group [71]. However, Cry34 proteins do share domain homology with aegerolysin-like proteins [72]. Cry35Ab1 is a member of a family of 44 kDa Bt proteins that share low sequence homology to Bt Cry36Aa1, *Bacillus sphaericus* mosquitocidal binary proteins

BinA and BinB [70], and *B. sphaericus* mosquitocidal binary protein, Cry49Aa1 [73]. It is interesting that Cry49Aa1 functions in combination with a 3-domain protein partner, Cry48Aa1.

As do other Cry proteins, the Cry34Ab1/Cry35Ab1 complex appears to function by disrupting the WCR midgut epithelium [74]. The proteins are also known to form ion channels in artificial lipid membranes [75]. Although the WCR receptors that bind Cry34Ab1/Cry35Ab1 have yet to be identified, Li et al. [76] demonstrated Cry34Ab1/Cry35Ab1 specific binding to WCR BBMV and a lack of competitive binding between Cry34Ab1/Cry35Ab1 and Cry3Aa, Cry6Aa, and Cry8Ba. Lack of competitive binding with the aforementioned single polypeptide Cry toxins, in addition to lack of sequence homology and the binary mode of action for Cry34Ab1/Cry35Ab1, suggested that Cry34Ab1/Cry35Ab1 can be combined with other Cry protein classes for resistance management. To this end, Cry34Ab1/35Ab1 have been coexpressed and developed for in-plant protection against WCR feeding damage and registered as maize event DAS-59122-7. These proteins have been combined by breeding DAS-59122-7 with maize events carrying Cry3 WCR traits to generate stacked traits for WCR IRM [45]. Further support for the suitability of combining Cry34Ab1/35Ab1 with other Cry protein classes was provided by the observations of Gassmann et al. [68] who demonstrated that field-derived western corn rootworm populations with reduced susceptibility to Cry3Bb1 corn were still effectively controlled by hybrids expressing Cry34Ab1/Cry35Ab1 proteins.

3 Insect Resistance Management

3.1 Principles of Bt Maize IRM

Protecting the durability of insect-protection traits in transgenic crops is a priority for technology developers, regulators, and end users [77]. The first commercialized traits used Bt proteins, such as Cry1Ab, Cry1Ac, and Cry3A that were similar to the active ingredients present in sprayable Bt formulations and were used as organic pesticides to protect a wide range of crops from injury by Lepidopteran pests. Therefore, protecting the durability of Bt proteins under greatly expanded use in transgenic crops was a priority, especially for organic food producers who rely on Bt formulations as one of their few options for insect pest management [78]. Because of their high efficacy, season-long Bt protein expression, and use in large-scale field crops, there is the potential for high levels of selection pressure for resistance [79]. Insect pests can evolve resistance to chemical insecticides under intensive use and there are examples of Bt resistance developing in the field through the use of sprayable formulations [80, 81].

Several options for resistance management for Bt crops were considered from a theoretical standpoint [79]. Production of the active ingredient within plant tissues

enabled more consistent and uniform exposure of the pests to the insecticidal proteins than could be achieved with traditional insecticide applications. The "high dose-refuge" approach provided a good fit for many of the key pest species targeted by the first Bt crops [82]. This approach relies on the Bt protein being produced at a concentration 25 times higher than that needed to kill 99 % of susceptible insects, with the expectation that there would also be high mortality (>95 %) of insects that are heterozygous for resistance alleles [83]. An additional assumption of this strategy is that resistance would be controlled by a major locus with two alleles: the wildtype conferring susceptibility and rare alleles conferring resistance. When coupled with a refuge of unprotected host plants, a low frequency of homozygous-resistant adults emerging from the Bt field mate with homozygous susceptible adults from the refuge field to produce heterozygous offspring. Because of the high dose, their offspring are killed by the Bt crop [79]. Therefore, the resistance allele frequency in the population rises very slowly.

Although the "high dose-refuge" approach can be highly effective in theory, and there is evidence that where properly implemented resistance has been successfully delayed [84], there are limitations to its general applicability [85]. When looking across different proteins, crops, and pests, high dose is the exception rather than the rule. Only for those species that are naturally highly sensitive to Bt proteins, such as *Ostrinia nubilalis* (European corn borer), *Heliothis virescens* (tobacco budworm), *Pectinophora gossypiella* (pink bollworm), and *Diatraea saccharalis* (sugarcane borer), can the high dose criteria reasonably be met [82]. For other species that are less sensitive to Bt proteins, high dose may not be achievable. *Diabrotica* species fall in the latter group and Bt maize producing Cry3Bb1, Cry34Ab1/35Ab1, or mCry3A have all been shown to allow measurable survival to adult even though they cause substantial larval mortality and provide high levels of root protection [86–90]. In addition to challenges achieving high dose, assumptions around the genetics of resistance appear not to hold universally. Many cases of resistance to Bt proteins, especially those associated with high levels of resistance, do indeed appear to be monogenic and recessive [91–94], however, others are more complex [95–97]. *Diabrotica* colonies selected against Bt corn show incomplete resistance, codominance, and may well be polygenic [87, 98, 99]. On the other hand, fitness costs are often associated with field-relevant resistance to Bt proteins [100] which are expected to help delay the onset of field resistance.

Perhaps the biggest challenge for the refuge-based IRM for many pests is reliable implementation of the refuge [101, 102]. In contrast to polyphagous pests for which a range of crop and wild plants can serve as refuge from Bt selection, for western corn rootworm, corn is the primary host plant and other hosts do not allow sufficient survival to adult. Adult dispersal of western corn rootworm is also thought to be limited [9], and neighboring farms may not reliably provide unselected adults that can mate with resistant insects in Bt fields. In these situations, growers must plant a portion of their fields to non-Bt hybrids. Non-Bt refuges represent short-term costs for farmers. From purchasing to planting, growers of Bt corn must consider the hybrid and location of their refuge corn. Because of limited

adult dispersal for corn rootworms, under the terms of the US EPA's registrations of rootworm-protection traits, the refuge must be planted within the same field as the Bt corn or in a neighboring field, limiting a grower's flexibility. By definition and design, refuge corn is vulnerable to root injury by corn rootworm larvae and represents yield and financial risk for growers. With the recent high commodity price of corn, the financial risk is considerable. These logistical and financial drivers limit refuge implementation. Recent data from technology providers [102] indicate that up to 10 % of corn growers fail to plant any refuge and 30–40 % of corn rootworm-protected Bt corn fields do not have an associated refuge within the required distance.

Laboratory selection experiments confirm that repeated exposure of corn rootworm populations across several generations can select for genotypes with greater survival on Bt corn [87, 98, 103] although in each case the laboratory resistance is incomplete. Furthermore, recent reports suggest that field resistance is developing to Cry3Bb1 Bt corn in some locations [68, 69]. Gassmann et al. [68] reported that the progeny of corn rootworm adults collected from Cry3Bb1-expressing corn fields showing unexpected root injury were on average better able to feed and survive longer on Cry3Bb1 corn than were the progeny of rootworm adults collected from non-Bt corn fields. This is consistent with Darwinian expectations ("survival of the fittest") and provides evidence that survival in less-than-high-dose situations can be associated with heritable changes in susceptibility that may be a lead to field resistance. Anecdotal reports suggest that fields that have been planted with corn producing the same Bt protein active against corn rootworm larvae for multiple years are vulnerable to increasing corn rootworm populations and root injury [104].

These concerns are leading technology providers and crop advisors to adjust their recommendations for long-term corn rootworm management to more explicitly include alternative tools such as rotation to corn rootworm non-host crops, soil- and foliar-applied insecticides, switching to Bt corn hybrids that produce a different rootworm-active Bt protein, and the adoption of Bt corn lines that produce more than one rootworm-active protein [104]. The last option, in particular, represents a particularly powerful resistance management tactic. Whereas crop rotation and insecticides are likely to be implemented on both Bt and non-Bt corn fields (to reduce local populations), corn lines with multiple active Bt proteins ("pyramided trait products") against corn rootworm require refuge corn to continue to be planted, so that a portion of the population escapes selection for resistance.

3.2 Theory of Pyramiding

Based on criteria that are expected to make pesticide mixtures with different mode of action more durable than continued use of a single mode of action [105], pyramided Bt traits in crops provide greatly extended durability. In pyramids, each

trait alone can be sufficiently high to provide effective control, and each component can be expressed throughout the larval feeding period of the target pests and in all tissues consumed ensuring continuous exposure. Cross-resistance among Cry proteins is more common and imparts greater levels of resistance among closely related Cry proteins than among more distantly related toxins [80, 96]. Because larvae that are heterozygous for resistance to one of the Bt proteins remain susceptible to the second, the refuge strategy is highly effective even in the absence of a "high dose" of either protein [48]. The presence of a small proportion of susceptible plants, recessive resistance, and redundancy in the mortality factors greatly extend the durability of two traits when pyramided compared with simultaneous or sequential deployment of the two traits individually [106].

Simulation models such as those by Gould [106], Gould et al. [107], Roush [48], Onstad and Meinke [108], Ives et al. [109], and Gryspeirt and Grégoire [110] suggest that pyramids can increase the number of generations until resistance occurs 25- to >100-fold compared with a single trait. Onstad and Meinke [108] showed that resistance evolution in *Diabrotica* is generally delayed by pyramided traits in maize compared with two single traits deployed sequentially. Ives et al. [109] similarly found that pyramided traits which cause low survival of insects heterozygous for resistance alleles can provide hundreds of generations of durability with very small (2–5 % of crop acreage) refuges. Greenhouse studies by Zhao et al. [111] have confirmed the conclusions of simulation models using broccoli lines expressing one or two Bt toxins and *Plutella xylostella* (diamondback moth).

The reduced refuge size associated with the pyramided traits has allowed the adoption of a natural refuge for Bt cotton in the southern United States such that the structured refuge requirement previously in place for single-gene cotton has been removed across most of the US cotton belt [112]. Similarly, planting limits for Bt cotton in Australia have been relaxed for Bollgard II. The US EPA and Canadian Food Inspection Agency, which have oversight of IRM programs for transgenic insecticidal crops in their respective countries, have also reduced the refuge size requirement for pyramided trait products compared with single-trait products in maize. For example, SmartStax® maize, which contains pyramided traits against corn borers and corn rootworms, requires a 5 % refuge rather than the 20 % required for single traits in Canada and the US corn belt. Reducing the burden of planting refuges is expected to encourage growers to adopt pyramided trait products in place of single-trait products [113] and improve their compliance with the refuge requirements.

Perhaps the most important change in refuge deployment enabled by the pyramiding insect protection traits and reducing required refuge sizes to 5 % has been the launch and adoption of corn seed products that contain a blend of Bt and non-Bt seed [112, 114]. Blended refuge products overcome one of the key obstacles to effective IRM: grower implementation of the refuge requirements. By blending refuge seed with the Bt seed, responsibility for refuge deployment reverts to the seed producer who can ensure not only that the correct refuge percentage is present but also that the refuge hybrid is an appropriate match for the Bt hybrid in terms of agronomic properties and seed quality. In addition, blended refuge products ensure

that the refuge seed is planted at the same time as the Bt seed and is planted in the same location, rather than on inferior quality land. Blended refuges also promote mating between susceptible and resistant insects that survive in the field, reducing the potential for spatial separation to promote mating among resistant insects. The main concern raised for blended refuges, which prevented their adoption for single-trait products, is that larval movement among refuge and Bt plants can reduce their effectiveness for IRM. Dispersing larvae may experience a diluted dose of the Bt proteins, potentially increasing the fitness advantage of resistant heterozygous insects over susceptible insects. At the same time, the proportion of unselected insects surviving in the refuge may be reduced. Mallet and Porter [115] and Davis and Onstad [116] showed that these factors can be very important in simulation models.

With pyramids and reduced refuge, these concerns are reduced and become outweighed by the benefits of refuge compliance and adult out-crossing [114]. Because insects would need multiple resistance alleles to overcome the Bt trait, the fitness advantage of heterozygotes derived from feeding in a blended refuge field is reduced. Furthermore, the smaller refuge percentage permitted through pyramiding reduces the proportion of larvae that encounter refuge plants. Finally, the large IRM benefit of pyramids compared with single-trait products means that even reduced effective refuge arising from larval movement provides superior durability.

3.3 Limitations of Current Pyramids

Pyramiding for resistance management is most effective if neither protein is simultaneously deployed as a single trait [117] and field resistance to each of the insecticidal proteins has not already developed. For corn rootworm-active Bt corn products today, each of the components of pyramids currently commercially available (Cry3Bb1, Cry34Ab1/Cry35Ab1, mCry3A) was first available as a single rootworm-protection trait product. Today, single-trait versions are still produced and selection for resistance in fields where these are grown is expected to lead more rapidly to resistance development than in fields where pyramided products are grown. Furthermore, indications that field resistance is already developing in some fields to Cry3Bb1 [68] suggest that pyramids that include this protein may not be as durable as intended in affected fields. If such resistance also provides cross-resistance to related proteins (e.g., mCry3Aa), pyramids that include both proteins may also be weakened. Finally, all of the pyramids currently available include Cry34Ab1/Cry35Ab1, leading to concerns about the durability of this protein [118].

Experience and models suggest that continued reliance on a narrow range of active ingredients, although preferable to a single active ingredient, will in time result in reduced efficacy and economic damage. Finding additional or replacement rootworm-active ingredients is an emerging priority, with an emphasis on novel modes of action for which cross-resistance with the existing proteins is unlikely.

Including one or more active ingredients that reduce or eliminate adult emergence may provide the greatest benefits by greatly reducing the potential survival of insects that are completely or incompletely resistant to other active ingredients.

4 New Modes of Action for Transgenic Control of Western Corn Rootworm

4.1 Candidate Insecticidal Proteins

In addition to Cry3 and Cry34Ab1/Cry35Ab1 proteins commercialized for WCR control, several classes of insectidal proteins derived from *B. thuringiensis* have been reported to be active against WCR. Candidate Bt toxins with activity on WCR include members classified as Cry, Cyt (cytolitic), and Vip (vegetative insecticidal protein) [41]. Collectively these proteins comprise a wide range of sequences from distinct homology groups that suggest new modes of action different from Cry3 or Cry34Ab1/Cry35Ab1. As such, these Bt toxin classes are the subject of intensive research efforts to discover novel candidates for WCR control in transgenic maize.

4.1.1 Cry6Aa1

Cry6Aa1 is a 54 kDa single chain polypeptide with no primary sequence homology compared to three-domain Cry toxins. Cry6Aa1 has dual pesticidal activity against WCR larvae [119, 120] and nematodes including *Meloidogyne* spp. [121, 122]. Cry6Aa does not share WCR brush border membrane binding sites with Cry34Ab1/Cry35Ab1 [76] and is therefore a suitable candidate for insect resistance management strategies that incorporate multiple modes of action.

4.1.2 Cry7 and Cry8 Toxins

Members of Cry7 and Cry 8 classes have been reported to be active on a range of Coleopteran pests [40]. Cry7 and Cry 8 proteins are three-domain toxins based on their primary sequence homology and conserved sequence blocks that are similar to other Cry toxins [38]. Both Cry7 and Cry8 classes contain multiple members with diverse sequences; at least 9 Cry 7 toxins and 24 Cry8 toxins are listed on the Bt nomenclature website [41]. The rich diversity of sequences in the Cry7 and Cry8 classes makes them attractive sources of candidate proteins to screen for activity against WCR. Indeed, a Cry7-type protein designated AXMI-28 [123] and a Cry8B protein [124] have been described as active against WCR. Cry8Ba does not share binding sites with Cry34Ab1/Cry35Ab1 [76].

4.1.3 Cyt1Ba1 and Cyt2Ca Toxins

Cyt toxins [125] are a subclass of Bt insecticidal crystal proteins that are named for their general cytolytic activity. Two Cyt toxins, Cyt1Ba [126] and Cyt2Ca1 [127] have been reported to kill WCR larvae.

Cyt toxins have a mode of action that is different from Cry toxins. Although Cyt toxin mode of action has not been reported in WCR, information from other insects suggests that Cyt toxins are potential candidates for transgenic expression for WCR control. Three Cyt protein structures have been solved that have a similar overall fold wherein two outer layers of alpha helix hairpins surround a beta sheet [128–130]. Cyt toxins function through interactions with non-saturated membrane lipids including phosphatidylcholine, phosphatidylethanolamine, and sphingomylin [131]. Cyt toxins are proposed to form multimeric pores or exert their insecticidal effect by a less-specific detergent mechnanism [132]. Cyt1Aa is well known for synergizing *B. thuringiensis* subspecies *israelensis* (Bti) mosquitocidal Cry toxins, that is, Cry4Aa, Cry4Ba, and Cry11Aa, and overcoming resistance to these toxins in mosquitos [133–135]. Furthermore, Cyt1Aa is toxic to the cottonwood leaf beetle, *Crysomela scripta*, and is able to overcome resistance to Cry3Aa in this pest [136].

4.1.4 Sip1A

Secreted insecticidal protein (Sip1A) is a novel insecticidal protein identified by screening culture supernatants from *B. thuringiensis* strains for activity against Coleopteran pests [137]. The approximate 38 kDa Sip1A protein is active on the larvae of the Colorado potato beetle, southern corn rootworm, and WCR. Sip1A lacks protein sequence homology with Cry toxins but has a low degree of sequence similarity with the 36-kDa mosquitocidal Mtx3 protein of *B. sphaericus* [137]. Mtx3 shares sequence similarity with *Pseudomonas aeruginosa* cytotoxin and to *Clostridium perfringens* alpha-toxin [39]. This is at least suggestive that Sip1A might belong to this class of pore-forming toxins.

4.1.5 Vip1 and Vip2

The soluble vegetative insecticidal proteins Vip1Aa and Vip2Aa from *Bacillus cereus* AB78 culture supernatants are arranged in a transcriptional operon and encode proteins of approximately 100 and 52 kDa, respectively. Vip1Aa and Vip2Aa act together as a binary toxin that is highly potent against WCR. Vip1Aa is processed at its N-terminus to an 80-kDa membrane binding protein that functions in a multimeric state to facilitate translocation Vip2Aa to the cytoplasm [138, 139]. Vip2Aa is an ADP-ribosylase with a high degree of sequence and structural similarity to the enzymatic domains of CdtA of *Clostridium difficile* and iota toxin of *Clostridium perfringens* [39, 140]. Vip2 consists of two structurally similar domains of limited sequence identity, with the C-terminal domain having

the enzymatic activity [140]. Given these structural similarities Vip2Aa likely targets actin to block polymerization leading to loss of the actin cytoskeleton and eventual cell death. Vip1Aa is homologous to the CdtB toxin component of *C. difficile*, the Ib component of *C. perfringes* iota toxin and the protective antigen of *B. anthracis*. These proteins bind receptors and translocate lethal factors, suggesting that Vip1Aa might function similarly [39]. The fact that Vip2Aa ribosylates actin could limit the utility of this binary toxin as an option for WCR control.

4.1.6 Bacterial Toxin Complexes (Tcs)

Several genera of insect pathogenic bacteria including *Photorhabdus* [141, 142], *Xenorhabdus* [143], *Serratia* [144], and *Yersinia* [145] produce toxin complex(es) (Tcs) with masses of approximately 1 million Daltons. The insecticidal activity of Tcs has generated interest in their potential use in agriculture as alternatives to Bt [146]. Tcs are characterized by at least three basic types of functional components: TcA, TcB, and TcC. Toxin complex from *P. luminescens* is highly active on *Diabrotica* spp. [147, 148]. Recent work demonstrated that *P. luminescens* TccC proteins are responsible for cell toxicity via ADP-ribosylation of actin and Rho-GTPases [149, 150].

4.2 RNA Interference

First described over 10 years ago in the nematode, *Caenorhabditis elegans* (Rhabditida: Rhabditidae), RNA interference (RNAi) refers to a set of related processes in which small regulatory double-stranded RNAs direct sequence-specific repression of gene expression [151–153]. This pathway has been implicated as a mechanism of defense against invasive nucleic acids from viruses or from mobile genetic elements, and has been conclusively shown to regulate gene expression in virtually all eukaryotic organisms [151, 152, 154, 155]. Widely recognized as one of the premier functional genomics research tools, RNAi has been used extensively in the postgenomics era to assign functions for genes annotated through small (expressed sequencing tags) or large (whole genome) scale sequencing efforts. Both the pharmaceutical and agricultural industries have recognized RNAi as a mechanism to control the expression of target genes for either therapeutic or pest control purposes, resulting in a diversity of potential applications.

In insects, the effectiveness of RNAi has been confirmed in a number of species but varies across different insect taxa and among different tissues [155, 156]. Most of the studies with insects have involved injection of long dsRNA directly in the insect hemocoel to achieve silencing which has become a routine method for assessing gene function. In the western corn rootworm, Alves et al. [157] documented the utility of RNAi in larvae by injecting second and third instars with dsRNA for genes that have previously been shown to exhibit clear RNAi

phenotypes in the Coleopteran model, *Tribolium castaneum* [158, 159]. Injection of dsRNA for the *D. v. virgifera* orthologues of *laccase 2* (*DvvLac2*) and *chitin synthase 2* (*DvvCHS2*) resulted in prevention of postmolt cuticular tanning for *DvvLac2*, and injection of *DvvCHS2*-specific dsRNA reduced chitin levels in midguts. Reduced expression of both *DvvLac2* and *DvvCHS2* was confirmed by RT-PCR and quantitative RT-PCR. These results suggest that RNAi-mediated gene knockdown is systemic in western corn rootworm larvae and provides a basis for utilizing RNAi as a functional genomic tool in rootworms.

The potential for RNAi to assess gene function in *D. v. virgifera* was exploited by Valencia et al. [160] who injected dsRNA for a putative endoglucanase (*DvvEngaseI*) that is believed to be involved in cellulose digestion into actively feeding second instars. A dramatic decrease in both *DvvEngaseI* transcript abundance and protein expression was reported confirming the knockdown of expression. However, the larvae injected with *DvvEngaseI* dsRNA showed only a slight decrease in weight gain and development time relative to control treatments. The authors concluded that suppression of a single cellulase gene by RNAi was insufficient to affect larval development significantly either because of the existence of multiple cellulases or the involvement of gut symbionts that may also facilitate cellulose digestion.

Although injection of dsRNA for functional genomics studies has been successful in a variety of insects including the western corn rootworm, uptake of dsRNA from the gut environment through oral exposure to dsRNA and subsequent down-regulation of essential genes is required in order for RNAi to be effective as a pest management tool [161, 162]. Although insects do not possess an RNA-dependent RNA polymerase (RdRp) common to plants and some lower invertebrates [161, 163], systemic RNAi through oral administration has been documented in a number of different species representing seven different orders [154]. The mechanism of systemic RNAi in insects is yet to be identified. A second potential obstacle to RNAi as a tool for pest management is the possible degradation of dsRNA in the insect gut [161]. However, the availability of transgenic plants that express dsRNA would provide continuous oral delivery and circumvent possible degradation.

The combination of systemic RNAi by oral exposure to dsRNA and transgenic plants that express dsRNA led to the first report of *in planta* RNAi in corn plants targeting *D. virgifera virgifera* larvae [164]. These authors describe a high-throughput in vivo dietary RNAi system to screen potential target genes for developing transgenic RNAi maize. A total of 14 genes from an initial gene pool of 290 exhibited larval control potential. One of the most effective double-stranded RNAs (dsRNA) targeted a gene encoding *vacuolar ATPase subunit A* (*V-ATPase*), resulting in a rapid suppression of corresponding endogenous mRNA and triggering a specific RNAi response with low concentrations of dsRNA. The authors also described that corn plants expressing dsRNA directed against the *V-ATPase* gene effectively reduced expression in rootworm larvae that fed on these plants and protected the plants from root damage, documenting for the first time the potential for *in planta* RNAi as a possible pest management tool.

Rangasamy and Siegfried [165] designed dsRNA for the same *V-ATPase* described by Baum et al. [164] and documented that oral delivery to adult rootworms could also induce reduced gene expression and protein synthesis and that mortality in the exposed beetles could be achieved within 14 days of exposure. The authors suggest that adults may provide a more effective developmental stage to screen activity of dsRNAs because they are easier to manipulate and can be induced to feed compulsively on an artificial diet by incorporating a natural feeding stimulant. The potential to target both adults and larvae with the same transgenic events may provide increased protection over technologies that target only larvae by minimizing egg deposition and larval damage in the subsequent growing season.

One of the factors that apparently influences RNAi efficiency in insects is the capacity of cells to take up dsRNA from the extracellular environment and spread the effect to neighboring cells [154]. Although the key steps in the process have yet to be confirmed for western corn rootworms, Bolognesi et al. [166] described a sequence of events that resulted in mortality after ingestion of a dsRNA designed from a gene (*Snf7*) which encodes an essential protein involved in intracellular trafficking. These events involve initial suppression of mRNA in midgut and some spreading to surrounding tissues within 1 day after exposure. By 3 days, suppression in the midgut had stabilized whereas suppression in tissues beyond the midgut continued to increase. By 5 days, DVSNF7 protein levels were significantly and equally reduced in midgut and other tissues equally and the onset of mortality was observed. The authors also reported that the minimum length of dsRNA for biological activity was approximately 60 bp and that 240-bp dsRNA containing a single 21-bp match to the target sequence was efficacious, whereas 21-bp short interfering RNA (siRNAs) were not. In addition, the uptake of dsRNA was dependent on fragment size as uptake of 240-bp dsRNA with a single 21-bp fragment embedded was evident in midgut cells whereas the 21-bp siRNA alone was not, suggesting that the lack of activity of siRNAs may be related to the ability of midgut cells to uptake the shorter fragments. These combined results provide important clues about the mechanisms of RNAi-induced suppression of gene expression and a basis for evaluating the specificity and potential for nontarget effects of RNAi-based control methods.

Given the recent reports of Cry toxin failure in field populations of western corn rootworms [68, 69], there is a clear and pressing need for alternatives to Bt toxins for rootworm management, and the development of *in planta* RNAi has taken on an increased urgency. Documenting efficacy of the technology is ongoing and regulatory considerations for RNAi-based insecticidal traits, such as the development of standardized environmental risk assessment, are still being developed [152, 162]. Considerations of how to evaluate sequence specificity, environmental fate, and exposure of nontarget organisms are still being developed. However, US regulatory agencies such as the Environmental Protection Agency and the Department of Agriculture have provided preliminary assessments [152] suggesting that data requirements for RNAi traits may be reduced based primarily on the lack of a plant-incorporated protectant, such as a protein toxin.

5 Summary, Conclusions, Outlook

The western corn rootworm is likely to continue to be a significant pest of concern in the US corn belt despite a history of control measures that have included chemical pesticides, crop rotation, and, more recently, transgenic maize lines containing WCR resistance traits. Transgenic maize that expresses toxins derived from *B. thuringiensis* has proven to be an effective and convenient means to control WCR and protect maize yield potential while reducing the cost of grower inputs and minimizing the environmental impact relative to traditional crop protection chemistry. This has driven an increase in grower adoption of WCR-trait technology to the extent that field-isolated resistance to the first WCR resistance trait, Cry3Bb1, is threatening the availability and utility of this toxin as well as the pyramided events that combine Cry3Bb with other toxins. WCR resistance to Cry3Bb1 is likely to increase selection pressure on Cry34Ab1/Cry35Ab1, the other commercialized WCR-trait with a truly unique mode of action different from three-domain Bt toxins.

It is clear that a robust IRM plan must be implemented in order to provide long-term durability for WCR trait technology. To be effective, WCR IRM must incorporate integrated pest management practices that include transgenic traits and effective refuge deployment as well as other rootworm management practices such as soil and foliar insecticides and crop rotation to protect trait durability. Refuge compliance will be simplified by the ability to provide the refuge maize lines as blended seeds, a scenario where the trait technology provider, rather than the grower, is responsible for refuge deployment. As discussed herein, combined or pyramided insect resistance traits with different modes of action are inherently more durable than single trait lines. Hence, the need for novel modes of action and WCR traits to enable effective trait pyramids is heightened.

The search for new modes of action includes intensive, genome-scale research for microbial-derived insecticidal proteins, such as Bt and other insect pathogenic microorganisms. The recent success in developing WCR-tolerant plants that express dsRNA targeting essential genes is encouraging and may provide an additional and unique mode of action to partner with existing traits and provide the seed industry with an additional tool for delivering robust IRM trait packages.

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Tribolium castaneum as a Model for High-Throughput RNAi Screening

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Abstract Coleopteran insects are a highly diverse and successful order, and many beetle species are significant agricultural pests. New biorational strategies for managing populations of beetles and other insect species are needed as pests develop resistance to chemical insecticides and Bt toxins. There is now an opportunity to use genome sequence data to identify genes that are essential for insect growth, development, or survival as new targets for designing control technology. This goal requires a method for high-throughput in vivo screening of thousands of genes to identify candidate genes that, when their expression is disrupted, have a phenotype that may be useful in insect pest control. *Tribolium castaneum*, the red flour beetle, is a model organism that offers considerable advantages for such screening, including ease of rearing in large numbers, a sequenced genome, and a strong, systemic RNAi response for specific depletion of gene transcripts. The RNAi effect in *T. castaneum* can be elicited in any tissue and any stage by the injection of dsRNA into the hemocoel, and injection of dsRNA into adult females can even be used to identify phenotypes in offspring. A pilot RNAi screen (iBeetle) is underway. Several *T. castaneum* genes with promising RNAi phenotypes for further development as mechanisms for plant protection have been identified. These include heat shock protein 90, chitin synthase, the segmentation gene hairy, and a matrix metalloprotease. Candidate genes identified in *T. castaneum* screens can then be tested in agricultural pest species (in which screening is not feasible), to evaluate their effectiveness for use in potential plant-based RNAi control strategies. Delivery of dsRNA expressed by genetically modified crops to the midgut of phytophagous insects is under investigation as a

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new tool for very specific protection of plants from insect pest species. The *T. castaneum* screening platform offers a system for discovery of candidate genes with high potential benefit.

Keywords *Tribolium castaneum* · RNAi · Pest control · Target genes · Transgenic plants

Abbreviations

RNAi	RNA interference
dsRNA	double stranded RNA
siRNA	short interfering RNAs
Bt	*Bacillus thuringiensis*
WCR	Western corn rootworm
RKN	Root knot nematode
HSP90	Heat shock protein
MMP	Matrix metalloproteinase

Contents

1 Introduction

More than 1 million insect species have been described, among which the Coleoptera (beetles) have enjoyed the greatest evolutionary success. Many are pests in agricultural systems, causing billions of dollars of damage every year. For example, the western corn rootworm (WCR, *Diabrotica virgifera virgifera*) is an economically important pest in the United States and an invasive species in Europe (Rice [60]. Beetles cause more than $1 billion in lost revenue per year in the

United States alone [83] and consume up to 20 % of harvested grain products in developing countries [52]. The use of insecticides to control beetles is becoming more challenging and expensive due to the emergence of resistant populations; these chemicals are also responsible for environmental pollution, harmful side effects on nontarget organisms, and a negative impact on human health, including an increased risk of cancer and immune system disorders [14, 102].

Insect pests can also be tackled using genetically modified (GM) crops, which were first grown commercially in 1996 [31]. Transgenic plants producing toxins from the soil bacterium *Bacillus thuringiensis* (Bt) were among the first GM crops to be developed [56]. The toxins are produced by bacteria as protein crystals (Cry) during sporulation, and are advantageous because they are toxic to certain groups of insects but do little or no harm to nontarget insects and vertebrates [16, 45]. The cultivation of Bt crops has led to significant reductions in the use of chemical insecticides [31], but resistant populations have emerged for several lepidopteran pests [8, 36, 39, 75, 77–80, 91] as well as WCR (Gassmann et al. [25]. Furthermore, certain insect pests are not affected by Bt toxins, including aphids, whiteflies, and leafhoppers, resulting in a demand for alternative biological control measures [18, 92].

Sequence-specific gene silencing by RNA interference (RNAi) has recently been suggested as a novel and promising approach for pest control because studies have shown an RNAi effect in insects triggered by feeding on plants containing dsRNA [9, 43]. RNAi occurs in a broad range of organisms including protozoa, invertebrates, vertebrates, fungi, and algae. This evolutionarily conserved process allows the specific targeting of any gene in a species-restricted manner, resulting in interference with growth, development, reproduction, and/or survival. Insect proteins required for these essential processes would make suitable candidates for RNAi-mediated plant protection if the corresponding dsRNA can be expressed in plants. To identify candidate target genes, high-throughput approaches for screening thousands of genes are required, but this may not be feasible in the pest species themselves.

The model organism *Tribolium castaneum* offers, among other advantages, a sequenced genome and a robust and strong RNAi response, which makes it suitable for large-scale RNAi screens. We recommend *T. castaneum* for high-throughput RNAi screens aiming to identify potential target genes in pest species for plant protection.

2 RNA Interference

The first indication for the existence of RNAi was reported by Napoli et al. [49]. Their aim was to produce transgenic petunias with a deeper violet color by enhancing the anthocyanin content of the petals. They transformed petunia plants with a chimeric chalcone synthase gene, encoding the key enzyme in flavonoid biosynthesis. Surprisingly, the overexpression of the chalcone synthase gene

suppressed anthocyanin biosynthesis by reducing chalcone synthase enzyme activity and yielded plants with white petals [49]. RNAi in animals was first observed in the nematode *Caenorhabditis elegans*, in which the injection of double-stranded RNA (dsRNA) resulted in the systemic and sequence-specific suppression of gene expression [24, 27]. The subsequent publication of the *C. elegans* genome sequence, rapidly followed by the human and fruit fly (*Drosophila melanogaster*), allowed RNAi to be applied in high-throughput investigations of gene function [15, 86].

2.1 The Mechanism of RNAi

RNAi is an ancient self-defense mechanism against virus infections [69, 94]. It is triggered by aberrant dsRNA, which is produced by the virus during replication and then degraded after recognition by the host cell. As well as its protective role against viruses, RNAi can also regulate transposon mobilization and enrich repetitive DNA to maintain genome integrity [95].

After the discovery of RNAi, intensive research revealed the underlying mechanism. When dsRNA enters the cell, it is recognized by an ATP-dependent ribonuclease known as Dicer, a dsRNA-specific member of the RNaseIII family of endonucleases [11]. Dicer processes the dsRNA into dsRNA fragments 21–23 nucleotides in length with two-base 3′ overhangs, known as short interfering RNAs (siRNAs) [11, 20, 105]. One of the two siRNA strands associates with the RNA-induced silencing complex (RISC), an enzyme complex comprising a number of proteins and RNAs, whereas the other strand is degraded [67, 90]. The siRNA serves as a guide to find the complementary target mRNA, which is cleaved after perfect base pairing with the siRNA [28, 105]. The core nuclease responsible for the cleavage and degradation of the target mRNA is Argonaute [44]. The intracellular process leading to gene silencing by RNAi is shown in Fig. 1.

2.2 Systemic RNAi

The efficiency and longevity of RNAi-mediated silencing differs according to the target species. In *D. melanogaster*, gene silencing induced by RNAi is restricted to the injected tissue, whereas in *C. elegans*, gene silencing induced by RNAi occurs throughout the organism, which is known as a systemic RNAi effect. The mechanism for systemic RNAi in *C. elegans* was clarified after identifying the systemic interference defective genes *sid-1* and *sid-2* [99]. The SID-1 protein is required to import silencing signals, whereas the SID-2 protein is localized in the intestine and is responsible for the exchange of dsRNA between the environment and the body through the midgut. Both genes are required for the success of feeding assays with dsRNA [98]. There is no *sid-1* ortholog in *D. melanogaster*, explaining the lack of

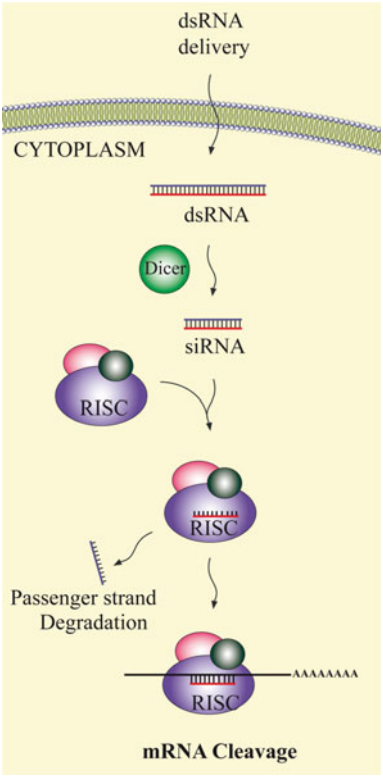

Fig. 1 The RNAi pathway is triggered by dsRNAs, starting with the processing of long dsRNA by the enzyme Dicer into siRNAs. In the following step, siRNAs are unwound, the passenger strand is degraded, and the guide strand binds to RISC. The active RISC is guided by the single stranded siRNA to target mRNA sequences and after perfect base pairing, the corresponding mRNA is degraded

robust systemic RNAi in this species [23, 62]. However, the systemic uptake of dsRNA has been confirmed in several other insects, including *T. castaneum* [15, 17, 40, 50, 86, 89]. Orthologs of SID proteins are not responsible for all cases of systemic RNAi, such as in the migratory locust (*Locusta migratoria*) [38]. Further studies are therefore required to determine the basis of systemic RNAi in these species.

3 RNAi-Mediated Plant Protection

As discussed above, current strategies to reduce the impact of insect pests suffer from numerous disadvantages, including off-target effects, environmental damage, and the emergence of resistant populations [81]. RNAi-mediated pest control is a relatively new strategy, which offers the opportunity to target individual or closely related species selectively, thus protecting nontarget organisms [97]. However, it may also be possible to design dsRNA molecules that target several related pests. Huang et al. [29] silenced the parasitism gene 16D10 in four species of root knot nematodes (*Meloidogyne* spp.) with a single dsRNA, showing the potential to achieve extended-spectrum resistance by targeting conserved sequence blocks.

Although microinjection is a powerful method for the induction of RNAi in animals, it is unsuitable for plant protection in the field. Instead, dsRNA must be delivered to phytophagous insects in a manner compatible with their lifestyle, generally via their diet. The uptake of dsRNA has been achieved using many techniques, including feeding *C.* elegans with bacteria expressing dsRNA [85], expressing dsRNA as an extended hairpin-loop RNA in *D. melanogaster* [33], and soaking embryos in RNA solution [19, 76]. In 2006, Turner and colleagues showed for the first time that RNAi can be triggered in insects (the light brown apple moth *Epiphyas postvittana*) following the oral delivery of dsRNA. Baum et al. [9] then silenced the vacuolar H^+ATPase in several coleopteran species (including WCR) by the oral administration of dsRNA. Successful RNAi following the oral delivery of dsRNA has now been reported for more than 20 species, including the insect orders Isoptera [110]; Hemiptera [4, 54, 55, 104, 106], Coleoptera [9, 12, 58, 97]; Diptera [37, 93, 97, 108], and Lepidoptera [10, 43, 84, 89, 97].

In the field, sprays containing dsRNA have proven effective for plant protection [107, 109], although spraying is only suitable for the aerial parts of the plant. For root pests, a possible solution would be the enrichment of soil surrounding the roots with dsRNA, as proposed by Artymovich [6]. However, neither method is currently cost effective because large amounts of dsRNA are required.

A suitable alternative is the development of GM crops producing dsRNA. This depends on the uptake of plant-derived dsRNA by insect gut cells and subsequent systemic RNAi effects, if the targeted genes are expressed beyond the gut epithelium [30]. Unfortunately, this mode of delivery may not be suitable for all species. In *Spodoptera litura*, the injection of dsRNA targeting a gut-specific aminopeptidase achieved an RNAi effect, but ingestion did not [57]. It is not possible to predict which dsRNA species can enter gut cells; therefore, each species and target gene must be tested on a case-by-case basis.

Despite these drawbacks, GM crops deploying an RNAi-based protection strategy have been successful against several pests, including nematodes [29, 74, 103], bacteria [21], and viruses [68], reviewed by Simon-Mateo and Garcia [70]. This approach has also been used to develop insect-resistant plants, such as transgenic maize expressing vATPase dsRNA for protection against WCR larvae [9]. Similarly, Mao et al. [43] identified a cytochrome P450 gene (CYP6AE14) from the cotton bollworm (*Helicoverpa armigera*), which is strongly expressed in the midgut and has a role in the detoxification of gossypol. Transgenic tobacco expressing CYP6AE14-specific hairpin RNA reduced the level of CYP6AE14 mRNA, which increased gossypol sensitivity in the larvae and delayed their growth.

One of the main advantages of RNAi-mediated crop protection is the low risk that resistance to the RNAi pathway will emerge because the core components are essential for metabolism and development. However, mutations in the dsRNA uptake machinery (such as the *sid* genes discussed above) is one potential route that could lead to resistance.

4 *Tribolium castaneum*

The red flour beetle *T. castaneum* (Fig. 2) is a global pest of stored agricultural products. Its use as a model organism began in the 1930s with studies on population ecology and pest control [71–73]. During the last decade, *T. castaneum* has emerged as a genetically tractable model organism for insect evolutionary and developmental biology. *T. castaneum* also has several advantages as a platform for RNAi screens.

T. castaneum has an ancient short-germ mode of embryogenesis, which is a characteristic of more primitive hemimetabolous insects [42, 82]. Another ancestral feature is the development of a larval head and legs, which are not present in *D. melanogaster*. These features make *T. castaneum* a more basal type of insect, and therefore results obtained using this beetle are more likely to broadly represent insects in contrast to *D. melanogaster* [65, 66].

Pest insects are often found seasonally in the field and are difficult to rear in the laboratory. In contrast, *T. castaneum* can be maintained at room temperature on wheat flour supplemented with 5 % yeast; it tolerates crowding as well as inbreeding. The generation time can be adjusted from 3 to 6 weeks based on the rearing temperature. Furthermore, adults experience long reproductive lives with high fecundity, even under laboratory conditions [71].

The robust RNAi effect is systemic in *T. castaneum* and can be elicited in any tissue and any stage by the injection of dsRNA into the body cavity [87]. The induction of RNAi in pupal or adult females causes an RNAi effect in the offspring, a phenomenon known as parental RNAi [15]. This allows the analysis of gene function in the next generation in the absence of physical effects caused by injection.

The *T. castaneum* genome was sequenced by the *Tribolium* Genome Consortium [88], providing the sequence data that is necessary for high-throughput RNAi screens and that is not available for most insect pests.

5 High-Throughput RNAi Screens

Genome-wide RNAi is a powerful approach because it allows the high-throughput investigation of gene function, which is particularly useful for the identification of genes with previously unknown roles in specific developmental processes or pathways. Potential off-target effects must be considered because some RNAi constructs can inactivate several genes with conserved sequence blocks. Therefore, the dsRNA sequences must be carefully validated to avoid homology with non-target mRNAs.

RNAi screens have been carried out in many different model systems, including the budding yeast (*Saccharomyces cerevisiae*), where 4800 genes were analyzed to identify those with an impact on longevity [32], and *D. melanogaster* cell lines, to

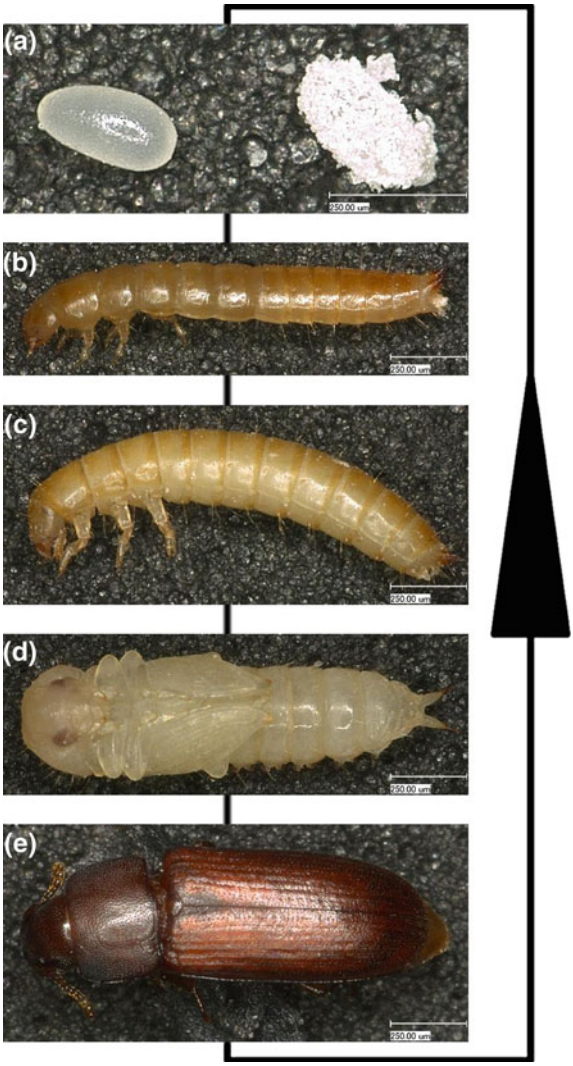

Fig. 2 Life cycle of *Tribolium castaneum*. **a** Egg. **b** Last-instar larvae (5–9 larval stages). **c** Prepupae. **d** Pupae. **e** Adult

investigate different signaling pathways [13, 22, 64, 96]. Whole-organism RNAi screens have also been carried out in *C. elegans*. For example, Ashrafi et al. [7] performed a genome-wide RNAi analysis and detected 417 fat-regulating genes, whereas Morton et al. [48] used a similar approach to identify 18 RNAi clones that caused embryo lethality.

A pilot RNAi screen (iBeetle) was initiated at the University of Göttingen and Erlangen to analyze the function of every *T. castaneum* gene [41]. This achieved the knockdown of approximately 5,500 of the 16,404 predicted genes within 1 year. The knockdown effect was analyzed in fifth-instar larvae and during embryogenesis by parental RNAi, exploring parameters such as metamorphosis

and fertility. The results are available on the iBeetle-Base (http://ibeetle-base.uni-goettingen.de) as described by Aronstein et al. [5].

6 Potential RNAi Targets

Preferential RNAi targets in pests include genes encoding proteins with essential developmental or metabolic functions. For example, heat-shock protein 90 (HSP90) is a ubiquitous and highly conserved chaperone involved in diverse signaling pathways [61]. Recently, *T. castaneum* HSP90 was shown to play a crucial role in postembryonic development, and silencing was found to be lethal at all developmental stages [35]. Further studies concerning the function of HSP90 have shown that knockdown produces females that are unable to complete oogenesis, suggesting a role during ovarian maturation [101]. Similar results were observed in *D. melanogaster,* in which homozygous *hsp90* mutations are lethal and reduced HSP90 activity causes sterility in females [53, 63]. These findings demonstrate that multifunctional HSP90 is an ideal target gene because RNAi causes pleiotropic effects.

Similarly, the suppression of vacuolar sorting protein Snf7 by feeding WCR larvae with the corresponding dsRNA caused the inhibition of larval growth and was ultimately fatal, suggesting that interference with basic insect cell functions in the field could be useful for plant protection [59].

Transgenerational RNAi provides additional opportunities to address pest infestation in the field by targeting insects prior to hatching. *T. castaneum* embryogenesis has been studied extensively, revealing many potential targets for agricultural pest management. For example, hatching can be prevented by knocking down chitin synthase, which causes the embryos to become twisted and enlarged and therefore unable to escape the egg casing [2]. Furthermore, Arnanda et al. [3] showed that head development can be disrupted by silencing the segmentation gene *hairy*, resulting in headless insects that are unable to feed on plants. Similarly, the matrix metalloproteinase MMP-2 plays a pivotal role in embryonic gut development. Thus, RNAi was lethal because the first-instar larva developed with an aberrant, twisted intestine [34] (Fig. 3).

The success of some insect pests depends on their ability to metabolize toxic phytochemicals that are produced by plants, offering additional targets for RNAi [26, 100]. For example, a cotton bollworm (*Helicoverpa armigera*) cytochrome P450 gene was targeted by producing dsRNA in transgenic plants, thus reducing larval tolerance towards gossypol, the major defense chemical produced by cotton [43].

It is likely that the ingested dsRNA will not spread beyond the gut epithelium in some pest insects, and the selection of targets would be restricted to genes encoding proteins that act in the intestine itself. The selection of suitable targets is therefore facilitated by the availability of transcriptomic data from the gut. In this

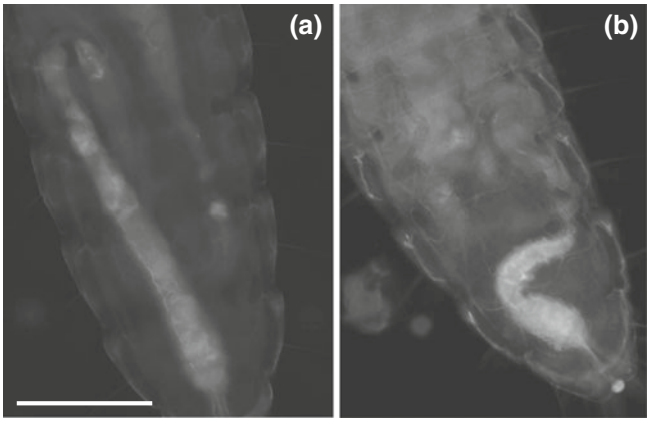

Fig. 3 *MMP-2* is a potential target gene because knockdown causes lethal gut defects in the progeny. **a** Autofluorescence of the abdomen from a wild-type neonate larva with a normal S-shaped intestine. **b** Autofluorescence of the abdomen from a first-instar larva subjected to *MMP-2* silencing, showing the twisted hindgut. Scale bars: 100 μm

context, the recently published gut transcriptome of *T. castaneum* will be beneficial for the selection of candidates for RNAi-mediated pest management [47].

7 Transferability From the Model to the Pest

The successful knockdown of particular target genes by RNAi in model organisms cannot be transferred directly to pests in the field. For example, the knockdown of HSP90 in the pea aphid *Acyrthosiphon pisum* has no effect on nymphal stages, which develop normally into adults. However, the birth of nymphs in the next generation is prevented, leading to embryos accumulating within the body cavity and (perhaps as a consequence) a shorter female lifespan (Will et al. in preparation). The knockdown of HSP90 in *T. castaneum* has a more dramatic effect because the protein is essential for normal postembryonic development. Silencing of HSP90 in *T. castaneum* also affected reproduction by disrupting oogenesis, although apparently by a different mechanism than that observed in aphids [101].

Based on the above, it is important to determine any potential effects of RNAi-mediated crop protection on nontarget organisms, particularly those that predate upon the targeted pest insect. Therefore, aphids that had ingested HSP90-specific dsRNA were fed to a natural predator, the ladybird beetle *Harmonia axyridis*, but there were no apparent off-target effects (T. Will, personal communication).

The optimal conditions for RNAi often vary among species and target genes, and this must be considered when searching for new candidates for crop protection. For example, RNAi against vATPase in the Colorado potato beetle (*Leptinotarsa decemlineata*) is more efficient than RNAi against the orthologous gene in

WCR [9]. Therefore, serial dilution experiments with dsRNA corresponding to each target gene are necessary to determine the efficiency of silencing [46]. Furthermore, sensitivity towards RNAi can differ during development. For example, in *T. castaneum*, the younger developmental stages show more sensitivity towards HSP90 silencing [34].

8 Conclusion

Plant-mediated RNAi is a promising approach for pest management, but high-throughput RNAi screens in pest species can be time-consuming and expensive. *T. castaneum* is a model beetle that is suitable for high-throughput RNAi screens. Therefore, prescreening in this species could help to identify new targets, leading to the identification of candidate genes in pest organisms [51].

Although the impact of gene silencing in *T. castaneum* may not always be transferable to orthologous genes in pest species, there are many examples showing the successful application of this approach resulting in the identification of useful candidate genes for pest control. For example, promising target genes characterized in *T. castaneum* were silenced in WCR causing similar phenotypes and could be used as a starting point for the further development of insect control strategies [1]. These findings show that RNAi-based pest control is a valuable alternative to chemical pesticides, complementing and extending existing field control methods.

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Aphid-Proof Plants: Biotechnology-Based Approaches for Aphid Control

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Abstract Aphids are economically significant agricultural pests that are responsible for large yield losses in many different crops. Because the use of insecticides is restricted in the context of integrated pest management and aphids develop resistance against them rapidly, new biotechnology-based approaches are required for aphid control. These approaches focus on the development of genetically modified aphid-resistant plants that express protease inhibitors, dsRNA, antimicrobial peptides, or repellents, thus addressing different levels of aphid-plant interactions. However, a common goal is to disturb host plant acceptance by aphids and to disrupt their ability to take nutrition from plants. The defense agents negatively affect different fitness-associated parameters such as growth, reproduction, and survival, which therefore reduce the impact of infestations. The results from several different studies suggest that biotechnology-based approaches offer a promising strategy for aphid control.

Keywords Agro-biotechnology · Antimicrobial peptide · Aphid · Pest control · Protease inhibitor · Repellent · RNAi

Abbreviations

AMP	Antimicrobial peptide
BPA	Body plan area
Bt	*Bacillus thuringiensis*
CaMV	Cauliflower mosaic virus
CC	Companion cell
dsRNA	double stranded RNA
Eβf	E-beta-farnesene
GM	Genetically modified
GUS	β-glucuronidase
PI	Protease inhibitor
RNAi	RNA interference

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SE	Sieve element
siRNA	small interfering RNA
SHP	Sheath protein

Contents

1 Interactions Between Aphids and Plants

In angiosperms, sieve tubes within the vascular bundles are conduits for nutrition (e.g. photoassimilates) and long-distance signaling. Sieve tubes are composed of longitudinally arranged modules of sieve elements (SEs) and companion cells (CCs). The SEs are connected to one another by sieve pores, which are modified plasmodesmata located in sieve plates at each end of the cell, embedded in the cell wall. Mass flow in the sieve tubes is created by a turgor difference between the source and sink ends [1, 2]. The phloem is subdivided into three functional zones [3]. In the collection phloem, photoassimilates accumulate in the SE/CC complexes within the minor veins of source leaves and are transported to the sieve tube ends in the release phloem of sink tissues such as fruits. The collection phloem and release phloem are connected by the transport phloem, which has a dual function. In the sieve tubes of the transport phloem, photoassimilates are transported from source to terminal sink, but some photoassimilates are released to support growth and maintenance of axial sinks along the pathway [4, 5].

The high nutritional content of sieve tubes makes them an attractive target for bacterial and fungal pathogens as well as insect pests. Therefore, SEs are equipped with defense mechanisms, including chemical components such as protease inhibitors [6] and physical components that lead to SE occlusion and thus the loss of mass flow. These physical mechanisms represent a special challenge for phloem-feeding insects, such as aphids. Ebbing mass flow in the sieve tubes prevents ingestion [7] because this is driven by the high pressure inside the sieve tubes [8]. In this context, there is increasing evidence that callose deposition onto sieve plates and sieve plate occlusion by phloem proteins (P-proteins) are important defense mechanisms against phloem-feeding pests such as aphids (e.g. [9–11].

Aphids are among the most important insect pests in agriculture. There are approximately 4400 aphid species, among which more than 250 are serious pests. In addition to direct damage caused by aphid feeding and the toxic effects of saliva components, the withdrawal of nutrients is detrimental to plant growth and development. Furthermore, aphids can transmit many plant viruses [12], and their excreted honeydew provides nutrition for sooty mold fungi, which can interfere with photosynthesis and reduce the market value of crops.

Aphids as well as other phloem-feeding hemipterans (e.g. whiteflies) have evolved specialized mouthparts (stylets) that penetrate through plant tissues to the sieve tubes, allowing the direct ingestion of sap. The feeding process begins directly after landing, when the aphid presents its labium (the mouthpart containing the stylets) to the surface. The labium is equipped with mechanoreceptors at its apex [13, 14] that can scan the leaf surface, presumably to detect the location of vascular bundles that often have overlying epidermal cells differing in shape from intervening epidermal cells.

The stylet pathway begins with the penetration of the epidermis and continues with stylet movement through the apoplast of the parenchymal tissue [15]. Gel saliva is secreted continuously during this process [16]. As the stylet advances towards the sieve tubes, they briefly perpetrate cortex and mesophyll cells, probably to orient the stylet inside the plant tissue [17]. Aphids take up a small amount of sap from these punctured cells for analysis by the precibarial sensilla located in the food canal between the base of the stylet and the sucking pump [18–20]. After penetrating an SE and identifying it as a source of nutrition, the aphid secretes watery saliva, which is followed by ingestion [21]. Although this feeding behavior has been described in detail by [22], the roles of the two types of saliva are not well understood but may play a key role in aphid–plant interactions [16].

Gel saliva is secreted onto the leaf surface at the penetration point and continues to be secreted as the stylet advances. It is secreted as a liquid but rapidly forms a solid salivary sheath that envelops the stylet. The most prominent saliva protein, the sheath protein (SHP), may be responsible for sheath hardening due to its high cysteine content. It is assumed that SHPs are solidified (gelled) by oxidation, through the formation of disulfide bonds among cysteine residues [16, 23–25]. Several functions have been proposed for the gel saliva: mechanical support of the stylet, protection of the stylet against molecular plant defenses (e.g. chitinases), lubrication to facilitate stylet movement, and the sealing of stylet penetration sites in the plasma membrane of plant cells [11, 26]. Watery saliva is also secreted during intercellular penetration [27], but in contrast to gel saliva it is secreted when the aphid stylet briefly punctures parenchymal cells and immediately before and during sap ingestion from SEs [28]. Recent proteome studies have identified several proteins (effectors) in watery saliva that potentially interfere with plant cell signaling cascades. Proteases and proteins of unknown function were also detected in the watery saliva, and their roles are the subject of intense research [23, 29].

Several authors have suggested that aphid saliva mediates insect-plant interaction by overcoming plant defenses before and after SE penetration, e.g. [26, 16, 25, 30]. In contrast to the large number of studies involving leaf-chewing insects

[31], direct evidence of the role of individual salivary proteins during aphid-plant interaction are rare [23, 29, 32, 33].

2 Control of Aphids

Chemical insecticides such as imidacloprid and dimethoate are used in conventional agriculture to control aphids, whereas azadirachtin from the Neem tree can be used for organic plant production [34]. An alternative approach is the use of beneficial insects (e.g. hoverfly, ladybeetle and brown lacewing) or entomopathogenic fungi [35]. Insecticides remain the most widely used control mechanism for aphids, even though the number of accredited insecticides has declined due to their negative impact on the environment. An additional problem with insecticides is the emergence of resistant aphid populations (http://www.pesticideresistance.com/). In the context of integrated pest management, biotechnology-based approaches offer an appealing alternative.

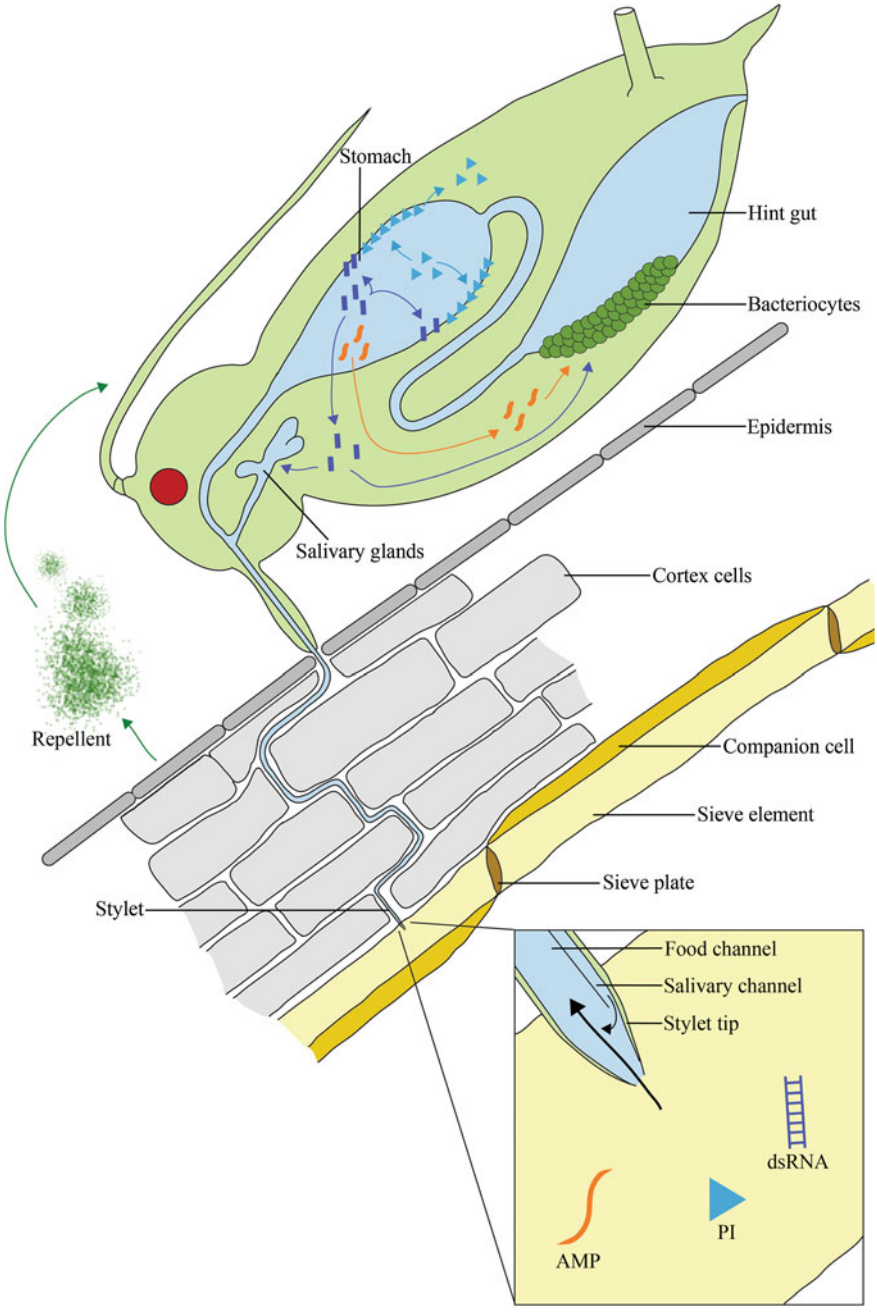
2.1 Biotechnology-Based Approaches

The use of genetically modified (GM) plants to fight insect pests [36–38] as well as fungal plant pathogens [39] has been established for more than 20 years, with most commercial insect-resistant GM crops expressing *Bacillus thuringiensis* (*B.t.*) toxins. Although these toxins are powerful and specific agents against Coleoptera and Lepidoptera [38], they do not affect phloem-feeding insects such aphids [40]. Therefore, alternative strategies are required for phloem-feeders, including the expression of protease inhibitors, RNA interference (RNAi), antimicrobial peptides, and repellents (Fig. 1). This requires a broad understanding of aphid biology as well as aphid-plant interactions to adapt such approaches to the specific properties of this pest.

2.1.1 Controlling the Expression of Defensive Agents in GM Plants

Effective pest control strategies using molecules expressed in plants must take account of the insect feeding strategy. Insects with chewing mouthparts, such as beetles, take up unspecific plant tissues material, whereas aphids have piercing-sucking mouthparts that are adapted for the withdrawal of sap from the xylem and phloem.

The *Cauliflower mosaic virus* (CaMV) 35S promoter is used to control transgene expression in many transgenic plants because it is regarded as a constitutive promoter, but the expression of a β-glucuronidase (GUS) reporter gene using this promoter indicates different levels of activity in different cell types [41]. High levels of


Stomach
Hint gut
Bacteriocytes
Epidermis
Salivary glands
Cortex cells
Repellent
Companion cell
Sieve element
Sieve plate
Stylet
Food channel
Salivary channel
Stylet tip
dsRNA
PI
AMP

◀ **Fig. 1** Overview of target localization for different defense-related agents—protease inhibitors (PIs), double-stranded RNA (dsRNA), antimicrobial peptides (AMPs), and repellents—used for aphid control in GM plants. Aphids penetrate plant tissue with their stylet (epidermis and cortex) and, after reaching the sieve elements, begin to ingest sieve tube sap. Aphids secrete pulses of saliva, produced in salivary glands and released from the salivary channel into the food channel, where it mixes with ingested sap. (E)-β-farnesene functions as an alarm pheromone and is released by specific glandular trichomes on the plant epidermis. It is perceived by chemoreceptors in the aphid antennae and acts as repellent. Repellents are already released prior to feeding following contact between the aphid and the trichomes. PIs, dsRNA, and AMPs are ingested as components of phloem sap. In the gut, PIs target proteases from the saliva and gut, thus interrupting digestion. Additional PI targets are located in the body cavity. Targets for siRNA, a product of dsRNA cleavage, were identified in the gut and salivary glands. There, siRNA induces the silencing of selected proteins required for aphid–plant interactions. Like PIs and dsRNA, AMPs can cross the gut epithelium and target bacterial endosymbionts that are located in the hemolymph or within bacteriocytes (e.g. *Buchnera aphidicola*), thus reducing aphid fitness

GUS activity were observed in the root pericycle cells and in the parenchymal cells of the xylem and phloem tissues in the stem and leaf. However, there was little or no GUS activity in the procambium, phloem, and cortex cells of the root; in the vascular cambium cells of stems; and in the majority of the cortex cells in the leaf midrib. Intermediate levels of GUS activity were observed in leaf mesophyll cells, certain ground tissue cells in the stem and leaf midrib, and in trichome and epidermal guard cells [41]. The activity of the CaMV 35S promoter is downregulated in older root areas and in syncytial feeding cells of nematodes [42]. Despite this inconsistent activity, the CaMV 35S promoter appears to be suitable for the expression of dsRNA to protect plants against coleopteran [43] and aphid pests [44].

The specific feeding strategy of aphids suggests that phloem-specific promoters would be more useful because they achieve targeted and potentially high-level expression in the phloem. This could increase the level of resistance towards phloem-feeding insects in GM plants by increasing the content of defense compounds in phloem sap while reducing the exposure of nontarget insects to the same compounds. Furthermore, this approach would also reduce the GM-associated resource investment by the plant by avoiding the expression of defense compounds in cells/tissues where they would never encounter the pest. The *SUC2* promoter that regulates the CC-specific *AtSUC2* sucrose-H^+ symporter gene is a good candidate because its activity is restricted to the phloem, with no differences between the source and sink tissues [45]. Imlau et al. [46] showed that green fluorescent protein expressed under the control of the *SUC2* promoter is transferred from the CCs via plasmodesmata to the SEs and is then transported along the sieve tubes. This provides proof of concept for the control of agents targeting phloem-feeding insect pests in GM plants. In contrast, the *SUT1* promoter, which regulates *StSUT1* (a sucrose H^+-cotransporter located in the phloem of potato plants), is active solely in the unloading phloem in sink tissues [47]. Therefore, it is unsuitable for the control of defense compounds because pests also infest source tissues such as mature leaves, and the transport of GM-based defense compounds from sink to source has yet to be demonstrated.

Promoters that are used for expression control of defense compounds and that are continuously active throughout the plant lifecycle can be regarded as inefficient because they produce these in the absence of infestation, and they can encourage the emergence of resistant populations. Therefore, promoters should ideally be inactive prior to infestation and/or wounding. Several promoters are inactive when tissues are intact but are activated by wounding, including the mannopine synthase (*mas*) promoter [48], the potato proteinase inhibitor II (*pin*II) promoter [49], and the *PR1-a* promoter [50]. The inducible *PR1-a* promoter is activated by salicylic acid, a chemical involved in wound-induced signaling in plants [50], and its production is triggered by aphid feeding [51]. The ideal promoter for the control of aphid resistance genes would therefore be chimeric, combining the functional elements of wound-inducible promoters (e.g. *PR1-a*) and phloem-specific promoters (e.g. *SUC2*). This would allow the development of GM plants with defense mechanisms triggered only by phloem-feeding insects such as aphids.

2.1.2 Protease Inhibitors

Protease inhibitors (PIs) are small molecules, peptides, or proteins that reduce or inhibit the activity of proteases by directly or indirectly blocking their active site or an adjacent exocite. PIs regulate the activity of endogenous proteases but can also act defensively against proteases secreted by pests and pathogens. They have been grouped into 48 families based on the sequence of the inhibitory domain [52]. As defense molecules, PIs ingested with phloem sap disrupt the digestion of proteins by insect proteases inside the gut, thus attenuating amino acid assimilation, slowing the growth of insects and reducing damage to the plant. Other targets in insects affected by PIs include water balance, molting, and enzyme regulation [53]. In non-GM plants, PIs are detected in storage organs and can be induced by insect feeding and pathogen infection [54]. The expression of trypsin inhibitors and other PI-like chymotrypsin inhibitors has already been achieved in the phloem of transgenic plants [55, 56].

Rhabé and Febvay [57] tested the toxicity of different proteins against the aphid species *Acyrthosiphon pisum* by artificial feeding *in vitro*. They found that the plant lectin concanavalin A was toxic and inhibited growth, whereas PIs were only effective at relatively high concentrations. A broader study of lectin and PI toxicity against five aphid species (*Aphis gossypii*, *Aulacortum solani*, *Macrosiphum euphorbiae*, *Macrosiphum albifrons* and *Myzus persicae*) revealed a dependence on the lectin/PI combination and aphid species [58]. Corcuera [59] suggested that naturally occurring PIs may defend barley against aphids, indicated by infestation induced accumulation of PIs against chymotrypsin and trypsin [60]. The authors used two aphid species (*Schizaphis graminum* and *Rhopalosiphum padi*) and observed that the amount of PI produced depended on the species and the number of aphids. PI activity was significantly greater in barley infested with *S. graminum,* probably reflecting the impact of each species; for example, *S. graminum* causes chlorosis around the feeding site, whereas *R. padi* does not [61]. Furthermore, PIs

significantly affected the survival of *R. padi* but had only a minor impact on *S. graminum*. PIs may also defend white cabbage cultivars and *Arabidopsis thaliana* against the aphid *Brevicoryne brassicae* [62].

The first GM plant expressing a PI for the control of plant-sucking insects was a tobacco (*Nicotiana tabacum*) plant expressing snowdrop lectin from *Galanthus nivalis* [63]. Tobacco plants are infested with the aphid *M. persicae*, but those feeding on the transgenic plants and on artificial diets containing the lectin showed reduced growth, survival, and reproduction. The insecticidal activity of snowdrop lectin was previously demonstrated against chewing insects in GM plants [64] and for planthoppers *in vitro* [65, 66]. Other PIs expressed in GM plants as defense compounds against aphids include oryzacystatin I in rapeseed [67] and eggplants [68], and a cysteine-PI from barley in *A. thaliana* [69]. These generally demonstrated similar effects to those described previously (i.e. reduced survival, growth, and reproduction), as well as a developmental delay. The use of PIs for aphid control therefore appears to be an effective strategy for pest management [63, 67–69].

Until recently, the target for PIs in aphids was uncertain because of conflicting data concerning the protease activity in the aphid gut [70]. Initially, aphids were considered to be unable to digest proteins in the sieve tube sap, thus relying on free amino acids as a nitrogen source [56]. However, an aminopeptidase and a cathepsin-L-like cysteine protease are thought to be immobilized in the gut of *A. pisum* [58, 71, 72]. Aminopeptidase, which represents 15.6 % of the total gut protein, may be a binding site for lectins [72]. An additional study identified cathepsin-B-like proteases in the *A. pisum* gut [70]. More recent findings indicate that several types of proteases, including metalloproteases, are present in the watery saliva of *A. pisum* [23, 29]. Because watery saliva is secreted into pierced SEs and mixes during nutrition uptake with the phloem sap in the stylet [16], plant-derived PIs target aphid proteases in two different environments, the sieve tubes and the alimentary tract. Additional targets for PIs may be present elsewhere in the aphid body because some PIs, such as oryzacystatin I, can cross the gut epithelium [67].

Despite the positive results achieved using different PIs against aphids, key considerations include the potential for aphids to adapt to PIs and the potential impact of ingested PIs on aphid predators and parasitoids. The overexpression of endogenous proteases could outcompete PIs and the expression of insensitive proteases could circumvent them, as previously seen in caterpillars and beetles [53]. A comparable observation was recently described for *M. persicae*, which upregulates expression of cathepsin B following PI ingestion [73]. Furthermore, oryzacystatin I is not only toxic towards the aphid *M. euphorbiae* but also to its parasitic wasp *Aphidius ervi* [74].

2.1.3 RNA Interference

RNA interference (RNAi) is a posttranslational RNA-mediated gene silencing process controlled by the RNA-induced silencing complex (RISC). RNAi is the major antiviral defense mechanism in both plants and insects [75, 76]. In insects,

the short interfering RNA (siRNA) pathway is the principal antiviral pathway and is considered to be part of the insect innate immune system [76].

Double-stranded RNA (dsRNA) derived from an exogenous source (e.g. a virus) or an endogenous source (e.g. pre-miRNA) is cleaved inside the cell by a ribonuclease III known as DICER to generate siRNAs or miRNAs 20–23 nucleotides in length with short tails [77, 78]. These are separated into single strands and the guide strand is integrated into the RISC complex [79], whereas the passenger strand is degraded. The siRNA or miRNA-RISC complex binds to its target mRNA resulting in cleavage (siRNA) or translational repression (miRNA) [78]. This process, when mediated by siRNAs, specifically reduces the abundance of target mRNAs [80].

Artificial exogenous sources of dsRNA can be provided by feeding or by the expression of hairpin RNA constructs in transgenic plants. The latter mechanism is termed host-induced gene silencing because the plant host delivers siRNA to the pest or pathogen [81, 82]. It is still unclear how exogenously administered dsRNA and siRNA enters insect cells [83].

Two early studies demonstrated that plants can be engineered to produce dsRNA, offering protection against specific insect pests. Baum et al. [43] transformed corn to produce dsRNA targeting the V-type ATPase A subunit mRNA, significantly reducing feeding damage by Western corn rootworm larvae (*Diabrotica virgifera*). Mao et al. [84] targeted the gut-specific cytochrome P450 gene of the cotton bollworm (*Helicoverpa zea*), which confers resistance to gossypol, a polyphenol defense compound produced by cotton plants. Bollworm larvae were initially fed on transgenic tobacco and *A. thaliana* plants expressing target-specific dsRNA, which made the insects sensitive towards gossypol present in artificial diets. The target specificity of dsRNA coupled with its ability to suppress genes that are critical for insect–host interaction or insect survival, for example, suggests that dsRNAs can be developed as highly specific pesticides, allowing the control of one or more specific insect pests without off-target effects [85].

In aphids, RNAi-mediated gene silencing has been achieved by injecting dsRNA or siRNAs into the hemolymph [86, 87] or by artificial feeding with dsRNA [85, 88]. In these studies, RNAi was used to investigate the function of proteins, such as the uncharacterized salivary gland protein C002 [33, 87] and a gut-specific aquaporin [88]. Jaubert-Possamai et al. [86] demonstrated that a single dose of dsRNA induces temporal silencing in aphids, with peak inhibition of 30–40 % target mRNA levels 5 days after injection, returning to normal 7 days after treatment. In this context, Mutti et al. [87] reported a 50 % reduction in the expression of a salivary gland protein. In *Tribolium castaneum*, a parental effect was observed in which the inhibition of target genes is transmitted to offspring [89], but no comparable studies have yet been carried out in aphids.

The proof of concept for transgenic plants delivering dsRNA to aphids resulted in the specific inhibition of Rack1 (located in the gut) and C002 (located in the salivary gland) in the green peach aphid *M. persicae* [44]. Both tobacco and *A. thaliana* were transformed with the silencing constructs, inducing up to 60 % silencing in the feeding aphids and reducing their fecundity. Surprisingly, silencing

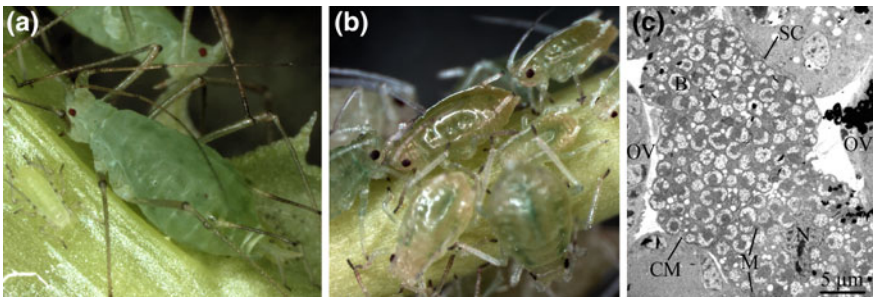


Fig. 2 **a** *Acyrthosiphon pisum* and **b** *Myzus persicae* are model aphids that have been used in most published studies, reflecting the availability of genomic resources for both species. *Buchnera aphidicola*, the primary bacterial endosymbiont of aphids, is located within bacteriocytes and is transmitted vertically to embryos. **c** *Aphis fabae* embryo bacteriocytes and surrounding tissues. Note the close packing of symbionts. *B Buchnera aphidicola* cell; *CM* cell membrane; *M* mitochondrion; *N* lobed nucleus; *OV* part of ovariole; *SC* sheath cell. (*Buchnera aphidicola* image kindly provided by Tom L. Wilkinson, University College Dublin, Ireland)

C002 did not reduce survival, as previously observed with *A. pisum* after siRNA injection [87]. This may reflect species-dependent differences or the impact of different application methods.

Experiments on aphids as a model for piercing–sucking pests suggests that the most promising RNAi targets are salivary proteins [33, 87] and gut proteins [88]. However, additional promising targets include transporters in the bacteriocyte plasma membrane, which are required for the transport of nutrients between the aphid and its obligate bacterial endosymbionts, such as *Buchnera aphidicola*. Most RNAi studies in aphids have focused on *A. pisum* and *M. persicae* (Fig. 2a, b) because the corresponding genome sequences are available, allowing the identification of RNAi target genes (IAGC [90]. The sequencing of additional species such as *A. gossypii*, *Diuraphis noxia*, *M. euphorbiae*, *M. persicae,* and *S. graminum* is in progress (IAGC [91, 92]; http://arthropodgenomes.org/wiki/i5K) and would broaden the scope of RNAi-based aphid control.

2.1.4 Antimicrobial Peptides

Peptide antibiotics are synthesized ribosomally in all organisms and in addition are produced enzymatically in fungi and bacteria. In eukaryotes, peptide antibiotics are termed antimicrobial peptides (AMPs) and generally comprise 12–50 amino acids. AMPs are active against Gram-positive and Gram-negative bacteria in different ways, according to their structure. Three main structural classes have been described: (1) linear α-helical peptides lacking cysteine residues; (2) peptides adopting a β-sheet globular structure stabilized by intramolecular disulfide bridges; and (3) peptides with an unusual bias for certain amino acids, such as histidine, glycine, proline, or tryptophan [93]. The production of peptides with

direct microbicidal activity is considered to be the most ancient mechanism of immunity. The formation of peptide-induced transmembrane pores in bacteria or other peptide-mediated mechanisms of membrane disruption abolishes the maintenance of membrane potential and causes bacterial cell death. Other AMPs have intracellular modes of action, such as the inactivation of bacterial DnaK [94].

AMPs represent the innate immune system, which is the only form of immunity in arthropods [95, 96]. Many insect species produce diverse AMPs [97, 98], but these are not present in aphids, nor do aphids produce components enabling the recognition and signaling of bacterial infection [99, 100]. The lack of an antibacterial defense response may reflect the close relationship between aphids and their endosymbiotic bacteria; for example, *B. aphidicola* is localized in specialized aphid cells known as bacteriocytes [101]; Fig. 2c). Additional facultative bacterial endosymbionts include different strains of *Hamiltonella*, *Serratia*, *Rickettsia*, and *Regiella* spp. [102]. Facultative endosymbionts may be intracellular and/or free within the hemolymph [103–105]. Aphids benefit from symbiotic bacteria because they convert nonessential amino acids in the phloem sap into essential amino acids [90], which are normally present at minimal levels [106]. Facultative symbiotic bacteria also confer resistance to parasitoid wasps [107], pathogenic fungi [108, 109], and heat [110, 111], as well as better performance on different host plants [112, 113]. The reliance of aphids on bacterial endosymbionts makes the latter a useful target for AMPs expressed in plants [114] based on the observation that eliminating different aphid bacterial endosymbionts using antibiotics reduces fecundity and delays aphid development, e.g. [115].

There has been one report thus far describing the influence of AMPs on aphids, using indolicidin as a model [116]. Indolicidin is a cationic AMP present in bovine neutrophils [117]; it shows activity against fungi [118] and bacteria such as *Escherichia coli* [117], which is closely related to *B. aphidicola*. Le-Feuvre et al. [116] demonstrated that the ingestion of indolicidin reduces the number of bacteriocytes in *M. persicae*, disrupts their structure, and reduces the number of bacteria, ultimately reducing the performance, survival, and reproduction of the aphids. Although antibiotic and AMP feeding generate distinct results, perhaps reflecting the secondary effects on gut cells or other internal tissues [116], these findings nevertheless indicate that AMPs produced by GM plants offer a promising experimental approach for pest control. Beyond that, proof of concept has been demonstrated for the control of fungal infections by AMPs expressed in plants, offering a new dimension to the defense system of plants that remain infested with unchallenged pests, e.g. [39, 119].

2.1.5 Repellents

Aphids detect odors via receptors in the primary and secondary rhinaria, which are antennal segments present in the Sternorrhyncha [120–124]. It has been suggested that the detection of plant volatiles is restricted to the primary rhinaria [120, 124, 125]. The overall response of these receptors to odors can be studied by

electroantennography, which measures the average output of antennal nerves to the brain for a tested odor [126]. Plant volatiles are used by aphids for long-range orientation [127] and responses have been recorded in species such as *S. avenae*, *Metopolophium dirhodum* [124, 128], *Aphis fabae* [122], *Megoura viciae* [129, 130], *A. pisum* [125], *B. brassicae,* and *M. persicae* [131]. Like other animals, aphids use pheromones for intraspecific communication, and these are also perceived by the antenna.

Pheromones are chemicals secreted into the environment to induce a social reaction from conspecifics. As well as aggregation and mating pheromones, chemicals such as (E)-7,11-dimethyl-3-methylene-1,6,10-dodecatriene (also known as E-β-farnesene or Eβf) function as alarm pheromones in aphids such as *R. padi*, *M. dirhodum*, *S. avenae* and *M. persicae* [132] and also in some beetles and wasps. Receptors for alarm pheromones are located in the two primary rhinaria in aphids [133]. The alarm pheromone is secreted by endangered aphids (e.g. in the presence of a predator) and induces others to stop feeding and escape, thus interrupting the feeding cycle and increasing alertness and the time spent walking or dropping off the plant at the expense of resource accumulation [134, 135].

Several plants, including wild potato species, have been shown to synthesize Eβf as a natural aphid repellent [136, 137]. In this context, the volatile is termed an allomone—that is, a substance that induces a reaction in a different species without any benefit to that species. Gibson and Pickett [137] suggested that the allomone is secreted by specific glandular hairs on the leaf surface and demonstrated that aphids remain a distance of 1–3 mm from the leaf surface during choice experiments. Nevertheless, the authors observed that not all aphids treated with air from Eβf-emitting potato species were disturbed during feeding on susceptible plants. As well as showing alarm responses, groups of aphids react to Eβf by producing a higher ratio of winged offspring (migratory morphs) after application [138], which has also been demonstrated in the field [139]. These observations suggest that plants producing aphid alarm pheromones benefit from a reduced number of feeding aphids and a higher ratio of winged offspring tending to leave the host plant [138]. The aphid resistance of a recently described melon line may reflect the same phenomenon [140], and it has been suggested as a strategy to produce aphid-resistant versions of economically-relevant cultivars [137].

Eβf also shows kairomonal effects by attracting *Adalia bipunctata* [141], *Coccinella septempuctata* [142], *Coleomegilla maculate*, *Hippodamia convergens*, *Harmonia axyridis* [143], the primary aphid parasitoid wasps *Aphidius uzbekistanicus* [144] and *A. ervi* [145], and the hoverfly *Episyrphus balteatus* [146]. This dual effect as a pest repellent and an attractant for beneficial insects increases the benefits of Eβf production by GM plants for aphid control. A recent study indicated that the dispersal of herbivore-induced plant volatiles affects insects to a range of 8 m from the release site [147], corroborating the idea that plant emitted volatiles affect a wide range around the release site.

Only one study has thus far shown the direct benefits of Eβf produced by GM plants [148]. *A. thaliana* producing Eβf were created by introducing the *Mentha x piperita* (peppermint) encoding Eβf synthase under the control of the CaMV 35S

promoter. Aphids showed more frequent alarm responses when exposed to a droplet of hexane containing entrained volatiles from a transgenic plant or to air from the headspace above Eβf-producing *A. thaliana* plants. Furthermore, the authors showed that the released Eβf attracted the parasitoid wasp *Diaeretiella rapae*, which spent more time on the transgenic plants than on comparable plants lacking the pheromone. There appeared to be no metabolic costs of Eβf synthesis because the transgenic plants showed no differences in growth or seed production compared to wild-type controls. Current work at Rothamsted Research (UK) focuses on the production of aphid-resistant wheat based upon the results of Beale et al. [148].

In contrast to Beale et al. [148], Kunert et al. [149] found that transgenic *A. thaliana* plants producing Eβf were not resistant to infestation by *M. persicae*, and did not affect reproduction or the ratio of winged and wingless offspring. The amount of Eβf produced by the plants was not influenced by aphid infestation [149]. The absence of a repellent effect may have reflected an adaptation to Eβf due to the continuous release by the transgenic plants. In contrast to the GM plants, wild-type plants release Eβf via glandular hairs in pulses, mimicking the Eβf emission of aphids when they are attacked by predators.

3 Testing Aphid Resistance in Plants

Developed GM plants are initially tested using molecular biology tools (e.g. quantitative PCR) to confirm transgene integration and the expression of the corresponding products and to compare plant lines produced by independent transformation experiments. GM pest/pathogen-resistant plants must then be tested for their efficiency against targeted pests/pathogens.

3.1 Aphid Fitness Parameters for Pest Control

Aphid fitness parameters such as development, body size, reproduction, and survival are relevant for plant infestation and thus are used to determine the efficiency of pest resistance. These parameters depend upon access to nutrition and its quality. The key parameter relevant to plant infestation by aphids is the remarkable rate of reproduction. Most aphid species show cyclical parthenogenesis under natural conditions with a switch from asexual to sexual reproduction. Reproduction begins approximately 1 week after birth; thus the development of aphids is rapid compared to similar-sized insects whose development lasts approximately 3 weeks. This reflects the so—called telescoping of generations in which aphid embryos begin to develop in their grandmothers. All these factors lead to a high rate of reproduction and make aphids ideal r-strategists with a total reproduction potential of several millions of progeny per season distributed over several generations.

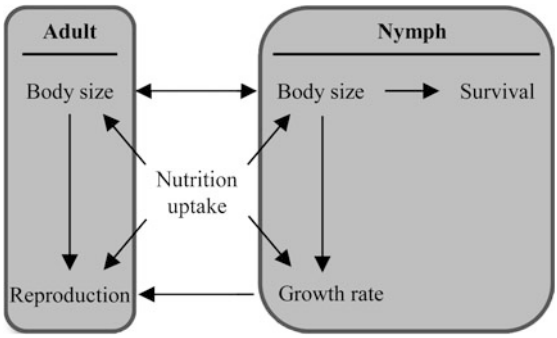


Fig. 3 Influence of nutrition uptake on different fitness-associated parameters and their interplay in aphid adults and nymphs

As discussed above, biotechnology-based approaches have a negative impact on parameters such as development and body size (Box 1) by reducing the intake of nutrition and thus the fitness of adults and offspring (Fig. 3). Nymphs with a low birth weight grow slowly and produce smaller nymphs in the next generation. Furthermore, slower growth increases the time to maturity and reproduction starts later in contrast to larger nymphs, reducing the total reproduction time over the lifespan of each aphid [150]. The final body size is also positively correlated with the reproductive weight [151, 152]. Larger and faster-developing nymphs also show higher survival rates and less parasitization by wasps than smaller nymphs [153]. The negative impact of reduced nutrition on reproduction implies that aphid control strategies do not necessarily have to focus on killing. Approaches that reduce infestation below an economically relevant level are also of interest because they follow the concept of integrated pest management.

Box 1: Measuring the body size and development of aphids

Two technical approaches are used to measure the size of adult aphids. Groups of up to 10 aphids can weighed and the mass of a single individual deduced. This is necessary because the small size of aphids of 1–10 mm (species dependent) means individuals weigh less than 1 mg, which makes accurate determination challenging. Alternatively or in addition, it is possible to measure the so-called body plan area (BPA) by taking images of single aphids using a microscope and a connected digital camera. Image analysis software can be used for size determination, which is calculated on the basis of a scale bar [154]. It is also possible to correlate the BPA with the developmental stage (larval stage 1–4 and adult).

3.2 Observation of Behavior Reveals the Mode of Plant Resistance

Aphid behavior provides additional insights into the interaction between host plants and pests, allowing observers to distinguish between aphids that are repelled by a plant, unable to access the plant, or have disrupted nutrition uptake. Three

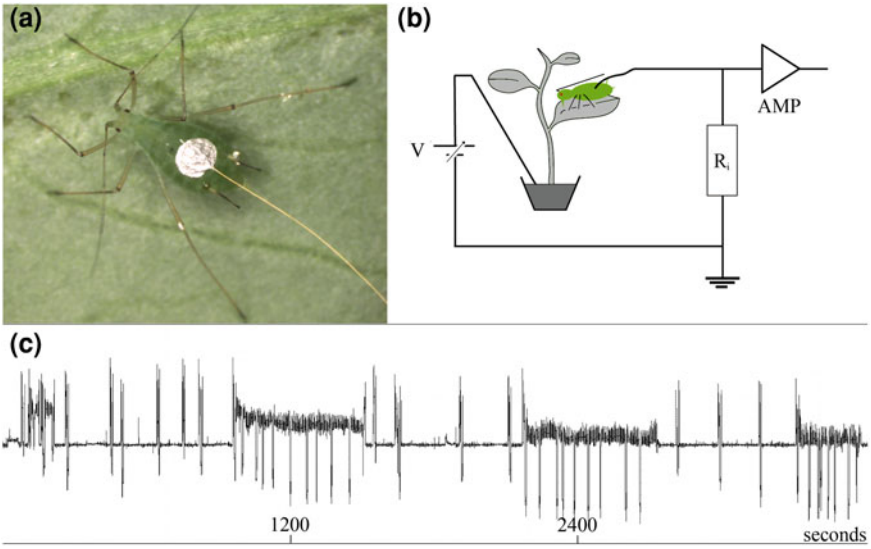


Fig. 4 Aphid behavior observed using the electrical penetration graph (EPG) technique. By attaching a thin gold wire with conductive silver glue to the dorsal abdomen (**a**), the aphid can be integrated in a direct current electrical circuit (**b**). The plant is integrated by inserting an electrode into the soil. The applied voltage is adjustable (V). The aphid and plant together represent a variable resistor. The input resistor (R_i) of the EPG amplifier has a value of 1 GΩ, about the mean value of the aphid. The measured signal is amplified 50-fold (Amp) and is recorded with a computer. (**c**) A 1-hour overview of an EPG recording. Specific waveforms in the EPG reveal information about stylet movement, salivary secretion, and ingestion, for example

main phases of plant-associated aphid behavior involve plant chemicals. The first phase is host plant identification by color and odor [155, 156]. This behavior can be studied with choice experiments using dual-choice chambers by which intact plants (resistant and susceptible) are offered to aphids [157]. Olfactometers, which can be designed as Y-track or four-arm models, are used as an additional tool to study the influence of plant repellents on aphids and the attraction of parasitoids *in vitro*, e.g. [141, 158].

In the second phase, aphids briefly penetrate epidermal and mesophyll cells and test the suitability of the plant as a potential host by taking a small sample of cell sap [159, 160]. The third phase is comparable to the second, but the ingested solution is sieve tube sap [16]. Varying artificial feeding setups, such as choice chambers [17, 161] or flow-through chambers [7], can be used to study aphid stylet orientation inside the plant as well as the influence of intracellular chemical and physical variations on feeding behavior. The electrical penetration graph (EPG) technique (Fig. 4) integrates the aphid and plant into an AC and/or DC electrical circuit [162, 163, 13, 14, 164], allowing feeding behavior inside the plant to be observed. The aphid and plant represent variable resistance in the electrical circuit that, in accordance with Ohm's law, influences the continuously recorded voltage [165]. Changes in resistance induced by the secretion of saliva or the uptake of

nutrients result in complex wave patterns that have been correlated with different patterns of behavior [21]. The EPG technique is a powerful tool to test the resistance of plants against piercing–sucking insects [166, 167] and to determine the site of resistance in the plant, such as in the epidermis, cortex, or phloem [168].

4 Future Perspectives for GM Plants

The first GM plants produced in 1983 by Fraley et al. [169]. It contained antibiotic resistance genes without any specific use in agriculture, but subsequent development focused on herbicide resistance [170] and pest resistance [171]. Although such first-generation GM crops with altered input traits remain the most widely grown, more recent developments include GM plants modified for output traits, such as β-carotene production in Golden Rice [172], and GM plants producing added-value compounds such as vaccines and antibodies [173]. New approaches in agro-biotechnology include RNAi, the expression of antimicrobial peptides, and the production of repellents for the control of aphids. The basis of this new generation of GM crops is the availability of more biological information and genome sequences from a higher number of pest organisms to facilitate target selection. Because these new approaches address physiological processes and basic modes of intraspecific and interspecific interactions among pests, their symbionts and their hosts, the development of resistant or tolerant pest populations appears unlikely. This is the basis of a new trend towards the development of tailor-made GM crops that can withstand one or several selected prominent pests in a respective habitat.

GM crops are currently grown on 160 million hectares [174], which represents 11.6 % of the total arable land area [175]. The five most important countries for production of GM crops are the USA, Brazil, Argentina, India, and Canada, and the four most prominent crops are soybean, corn, cotton, and rapeseed. The uptake of GM agriculture in developed and developing countries is expected to increase further, following a trend observed since the first GM crops were commercialized [174]. The consumer attitude towards GM agriculture differs between countries, with high acceptance in USA and Asia and a more cautious view in Europe [176]. It can be assumed that next-generation GM crops, developed according to knowledge-based principles, will increase overall acceptance and the economic potential of such crops. However, this will require better communication with the general public [177].

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