IRON NUTRITION IN PLANTS AND RHIZOSPHERIC MICROORGANISMS

Edited by
L.L. BARTON
J. ABADÍA

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Iron Nutrition in Plants and Rhizospheric Microorganisms

Edited by

Larry L. Barton
Department of Biology
The University of New Mexico
Albuquerque
New Mexico, USA

and

Javier Abadía
Department of Plant Nutrition
Estación Experimental de Aula Dei
Consejo Superior de Investigaciones Científicas (CSIC)
Zaragoza, Spain

Springer

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Front Cover Picture: This picture gives a visual idea of the effects of iron deficiency on the foliage of peach tree leaves, a crop that is often affected by this nutritional problem.

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Dedication

This book is dedicated to John C. Brown, Sei-ichi Takagi, and Arthur Wallace and to the memory of Horst Marschner. Not only were they pioneers in plant-iron relationships but they encouraged others to pursue this field of research.

Contents

Contributing Authors

Anunciación Abadía
Departamento de Nutrición Vegetal, Estación Experimental de Aula Dei, Consejo Superior de Investigaciones Científicas (CSIC), Apdo. 202, 50080 Zaragoza, Spain

Javier Abadía
Departamento de Nutrición Vegetal, Estación Experimental de Aula Dei, Consejo Superior de Investigaciones Científicas (CSIC), Apdo. 202, 50080 Zaragoza, Spain

Shah Alam
Ministry of Agriculture, c/o S.M. Jashim Uddin, 554 Monipur (2nd floor), Mirpur, Dhaka 1216, Bangladesh

Esteban Alcántara
Dpto. Agronomía, Escuela Técnica Superior de Ingenieros Agrónomos y de Montes, Universidad de Córdoba, Avda Menéndez Pidal s/n, Apdo. 3048, 14080-Córdoba, Spain

Ana Álvarez-Fernández
Departamento de Nutrición Vegetal, Estación Experimental de Aula Dei, Consejo Superior de Investigaciones Científicas (CSIC), Apdo. 202, 50080 Zaragoza, Spain

Larry L. Barton
Department of Biology, University of New Mexico, MSC03 2020, Albuquerque, New Mexico 87131-0001, USA

Petra Bauer
Institute of Plant Genetics and Crop Plant Research, Corrensstr. 3, D-06466 Gatersleben, and Botany, Saarland University, P.O. Box 151150, D-66041 Saarbrücken, Germany

Yvonne M. Bishop
Department of Biology, University of New Mexico, MSC03 2020, Albuquerque, New Mexico 87131-0001, USA

Jean-François Briat
Biochimie et Physiologie Moléculaire des Plantes. Centre National de la Recherche Scientifique (UMR 5004), Institut National de la Recherche Agronomique, Université Montpellier 2, École Nationale Supérieure d'Agronomie, 2 Place Viala, F-34060 Montpellier cedex 2, France

Thomas J. Buckhout
Applied Botany, Humboldt University Berlin, Invalidenstraße 42, 10115 Berlin, Germany

Françoise Cellier
Biochimie et Physiologie Moléculaire des Plantes. Centre National de la Recherche Scientifique (UMR 5004), Institut National de la Recherche Agronomique, Université Montpellier 2, École Nationale Supérieure d'Agronomie, 2 Place Viala, F-34060 Montpellier cedex 2, France

Dirk V. Charlson
Department of Biochemistry, University of Missouri-Columbia, Columbia, Missouri 65211, USA

Silvia R. Cianzio
Department of Agronomy, Iowa State University, Ames, Iowa 50011, USA

David E. Crowley
Department of Environmental Sciences, University of California, Riverside, CA 92521-0424, USA

Jason W. Ellsworth
Twin Falls R & E Center, University of Idaho, 315 Falls Avenue, Evergreen Building, P.O. Box 1827, Twin Falls, Idaho 83303-1827, USA

Dominique Expert
Laboratory of Plant Pathology (CNRS-INRA), Paris Grignon, 16 rue Claude Bernard, 75231 PARIS Cedex 05, France

Ferenc Fodor
Department of Plant Physiology, Eötvös University, Budapest, H-1518 P.O. Box 120, Hungary

Frederic Gaymard
Biochimie et Physiologie Moléculaire des Plantes. Centre National de la Recherche Scientifique (UMR 5004), Institut National de la Recherche Agronomique, Université Montpellier 2, École Nationale Supérieure d'Agronomie, 2 Place Viala, F-34060 Montpellier cedex 2, France

Michael A. Grusak
US Department of Agriculture, Agricultural Research Service, Children's Nutrition Research Center, Department of Pediatrics, Baylor College of Medicine, Houston, Texas 77030, USA

Mary Lou Guerinot
Department of Biological Sciences, Dartmouth College, Hanover, New Hampshire 03755, USA

Sophie Haebel
Center for Biopolymers, Potsdam University, Karl-Liebknecht-Strasse 24-25, 14476 Golm, Germany

Neil C. Hansen
Department of Soil and Crop Sciences, 1170 Campus Delivery, Colorado State University, Fort Collins, Colorado 80523-1170, USA

Bryan G. Hopkins
Cropping Systems, 1776 Science Center Dr., University of Idaho, Idaho Falls, ID, USA

Brenda Parson Hall
Department of Biological Sciences, Dartmouth College, Hanover, New Hampshire 03755, USA

Rüdiger Hell
Heidelberg Institute of Plant Sciences, Dept. IV Molecular Biology of Plants, University of Heidelberg, Im Neuenheimer Feld 360, 69120 Heidelberg, Germany

Alexandra Herbik
Applied Botany, Humboldt University Berlin, Invalidenstraße 42, 10115 Berlin, Germany

Takanori Kobayashi
Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

Gordon V. Johnson
Department of Biology, University of New Mexico, MSC03 2020, Albuquerque, New Mexico 87131-0001, USA

Von D. Jolley
Department of Plant and Animal Sciences, Brigham Young University, 253 WIDB, Provo, UT 84602, USA

Shigenao Kawai
Faculty of Agriculture, 3-18-8 Ueda, Iwate University, Morioka 020-8550, Japan

Carlos Lucena
Dpto. Agronomía, Escuela Técnica Superior de Ingenieros Agrónomos y de Montes, Universidad de Córdoba, Avda Menéndez Pidal s/n, Apdo. 3048, 14080-Córdoba, Spain

Juan J. Lucena
Sección de Química Agrícola, Facultad Ciencias C-VII, Universidad Autónoma, E-28049 Madrid, Spain

Satoshi Mori
Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

Naoko K. Nishizawa
Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

Roberto Pinton
Dipartimento di Scienze Agrarie e Ambientali, University of Udine, Italy

Immo Reinhardt
Applied Botany, Humboldt University Berlin, Invalidenstraße 42, 10115 Berlin, Germany

Elizabeth E. Rogers
Departments of Biochemistry and Nutrition Sciences, The University of Missouri, Columbia, Missouri 65211, USA

Adamo Domenico Rombolà
Dipartimento di Colture Arboree, Alma Mater Studiorum, Università di Bologna, Viale Fanin 46, 40127 Bologna, Italy

Francisco J. Romera
Dpto. Agronomía, Escuela Técnica Superior de Ingenieros Agrónomos y de Montes, Universidad de Córdoba, Avda Menéndez Pidal s/n, Apdo. 3048, 14080-Córdoba, Spain

Wolfgang Schmidt
Biological Science and Bioengineering Program, Faculty of Engineering and Natural Science, Sabanci University, 34956 Istanbul, Turkey

Randy C. Shoemaker
USDA-ARS, Corn Insect and Crop Genetics Research Unit, Department of Agronomy, Iowa State University, Ames, Iowa 50011, USA

Massimo Tagliavini
Dipartimento di Colture Arboree, Alma Mater Studiorum, Università di Bologna, Viale Fanin 46, 40127 Bologna, Italy

Zeno Varanini
Dipartimento di Scienze Agrarie e Ambientali, University of Udine, Italy

Marta Vasconcelos
US Department of Agriculture, Agricultural Research Service, Children's Nutrition Research Center, Department of Pediatrics, Baylor College of Medicine, Houston, Texas 77030, USA

Graziano Zocchi
Department of Crop Production, Section of Plant Physiology and Biochemistry, University of Milano, Italy

Preface

Animals including humans are highly dependent on plants to provide many different nutrients including iron in a useable form. Additionally, plants are used to support the growth of animals and obtaining high crop yields *via* optimal plant growth is an economic necessity. Thus, it is crucial to understand the role of iron in plant nutrition.

This book provides comprehensive reviews on topics of plant-iron nutrition that are being addressed by different laboratories around the world. As one can see, the area of plant-iron nutrition is highly interdisciplinary, involving scientists of various fields of knowledge. Plant biologists are needed to characterize iron translocation throughout the plant following root iron uptake and to examine the regulation of iron-stimulated activities that influence crop yield and quality. Plant geneticists are contributing to the area of plant-iron nutrition by developing model systems to aid our understanding of the complex activities of the individual plant. Soil chemists are examining the interactions between iron and various minerals and organic matter soil components in the root zone. Microbiologists are providing a crucial perspective on how the interactions between the plant and soil microorganisms are important in plant iron nutrition. Additionally, the cycling of iron in the terrestrial environment is being examined by ecologists and related scientists. While it may be ideal to systematically examine iron nutrition in a single plant species, research is influenced by local or regional requirements. As a result, research is conducted with many different plant species and at many different scientific levels. Thus, the reviews in this book address many different facets of plant-iron nutrition.

The organization of the chapters in the book starts with general nutritional aspects associated with iron and progress in characterization of

specific systems of plant-iron interactions. The first chapter addresses plants as an iron source for humans and it is followed by three chapters that deal with crop-oriented activities. Chapters 5 through 7 are soil oriented and include the impact of additives or amendments on iron nutrition. Chapters 8 through 10 examine the characteristics of symbiotic, pathogenic and mutualistic microorganisms on plant-iron nutrition. Chapters 11 and 12 focus on iron-uptake by plant roots while Chapters 13-16 examine iron transport and translocation throughout the plant. Chapter 17 reviews the activities related to ferritin in iron storage and Chapters 18-20 focus on changes caused in the metabolome and proteome associated with plant-iron stress responses. Chapter 21 provides and overview of the evolving technology that uses stable isotopes to follow iron metabolism and Chapter 22 discusses some of the genomic resources of different plant cultivars. Frequently, these chapters relate to plant-iron research covered in another chapter thereby offering a comprehensive discussion of the topic.

While the goal of these reviews is to summarize the current state of knowledge in the various areas, it also reveals the many unresolved questions concerning plant-iron nutrition. Research must not stop in this important area but new and innovative approaches are needed to gain a better insight into the topic. We hope that this book will stimulate research in the field of plant-iron nutrition with the continued exploration of plant biology and rhizospheric activities.

We are indebted to our colleagues who have made this book possible. The authors of each chapter have provided us with robust reviews even though they have numerous other demands on their time. The proofing and formatting of chapters by Sandra Barton, Ana Álvarez-Fernández and Victoria Fernández were essential for prompt completion of the book and we greatly appreciate their contributions.

Larry L. Barton and Javier Abadía

Chapter 1

STATUS AND FUTURE DEVELOPMENTS INVOLVING PLANT IRON IN ANIMAL AND HUMAN NUTRITION

MARTA VASCONCELOS and MICHAEL A. GRUSAK
US Department of Agriculture, Agricultural Research Service, Children's Nutrition Research Center, Department of Pediatrics, Baylor College of Medicine, Houston, Texas, USA. E-mail: mgrusak@bcm.tcm.edu

Abstract: Iron is an essential nutrient for humans and other animals, and must be consumed in adequate amounts to ensure proper growth and development, as well as good health of the organism. Dietary sources of iron can be divided into two types: non-heme iron, mostly provided by plant foods, and heme iron, present in animal foods. Heme iron intake is usually low for the majority of humans in many developing countries because of the high cost of animal products or due to cultural constraints concerning these foods. Heme iron intake also is low in most livestock, whose major source of dietary iron comes from forages and cereal crops. For these reasons, both humans and animals rely on plants as an important source of dietary iron. However, the iron concentration of plant foods varies greatly, and low concentrations in some common food sources make it difficult for humans and animals to meet daily dietary requirements when these foods are consumed in suggested amounts. Additionally, certain food components, such as phytate or tannins, can lower the bioavailablity of the iron that is in plant foods, thereby lowering its effective concentration even more. In order to improve the iron nutritional value of crop plants and consequently to improve human and animal health, several strategies are being utilized by plant scientists. These include: cultivar evaluation, plant breeding and marker-assisted selection, alteration of pathways of iron metabolism, and modification of iron bioavailability. In this review, we present the role that plant iron plays in the diets of humans and other animals, and discuss the strategies that can be employed to improve our plant-based food supply.

Key words: animal; bioavailability; human; iron; nutrition

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1. IRON ABSORPTION IN HUMANS AND ANIMALS

Iron is one of the most important micronutrients in the human diet, functioning as a component of a number of proteins, including enzymes and hemoglobin. It is crucial for optimal physical performance and cognitive development. At least four major classes of iron-containing proteins exist in the mammalian system: iron containing enzymes (hemoglobin, myoglobin, cytochromes), iron-sulfur enzymes (flavoproteins, heme-flavoproteins), proteins for iron storage and transport (transferrin, lactoferrin, ferritin, hemosiderin), and other iron-containing or activated enzymes (sulfur, non-heme enzymes) (Institute of Medicine, 2001).

Nutritional iron is usually divided into two types: heme iron, which is absorbed unaffected by other food components, and non-heme iron, which is envisioned as "free" or as weak complexes (Theil, 2004). Heme iron contributes only 10 to 15 percent of the total iron intake (1 to 3 mg/day) in diets of developed countries but may provide a substantial amount of the total absorbed iron. Where meat is consumed extensively, (e.g. Argentina and New Zealand) this contribution can rise to almost 50 percent. Heme iron intake is negligible for the majority of people in many developing countries, because of cultural constraints and the high cost of animal products (Bothwell *et al.*, 1989). For this reason, non-heme iron is the main source of dietary iron for most people in the world.

Generally, the iron content of the body is highly conserved, and iron balance is maintained by regulating absorption in the small intestine, where two principal mechanisms of iron absorption can be found on the luminal surface of enterocytes. The first mediates the absorption of heme iron, derived primarily from the proteolytic degradation of hemoglobin and myoglobin, and the second regulates the absorption of non-heme iron, extracted from plant and dairy foods (Institute of Medicine, 2001). Heme iron absorption is thought to occur via a vesicular transport process, whereby the entire heme complex is brought into the enterocyte, prior to the degradation of heme (by heme oxygenase) and the release of inorganic iron (Uzel and Conrad, 1998). The absorption of inorganic iron from non-heme sources involves a reduction and transport process. The human protein Dcytb is an iron-regulated ferric reductase that appears to utilize electrons donated from intracellular ascorbate (Atanasova *et al.*, 2005; McKie *et al.*, 2001). Ferrous ions generated by Dcytb are available for transport through the protein DMT1, which also is regulated by low iron status in the body (Gunshin *et al.*, 1997).

The total content of a given nutrient in any given food is not always a good indicator of its useful nutritional quality, because not all of the

nutrients in food are absorbed (Grusak and Dellapena, 1999). Iron in any food has a particular bioavailability, which is a function of its chemical form and the presence of food components (in that food, or in the diet) that either promote or inhibit its absorption (Abrams, 2004). Non-heme iron is generally less bioavailable than heme iron, due in part to the presence of tannins or phytate in some foods, which inhibit its absorption (Davidsson *et al.*, 1994). In fact, the bioavailability of iron in most plant sources is on the order of 5% (Consaul and Lee, 1983). On the other hand, foods also contain factors that stimulate the absorption of non-heme iron; these include organic acids (particularly ascorbic acid) and the poorly understood "meat factor" (Fidler *et al.*, 2004; Huh *et al.*, 2004).

2. IRON REQUIREMENTS

2.1 Iron requirements in humans

A large segment of the world's population does not ingest enough iron to meet daily dietary requirements. Therefore, iron deficiency and iron-deficiency anemia (IDA) are estimated to affect 30-50% of the world's population (Yip and Dallman, 1996), being especially prevalent in developing countries where food intakes can be severely low. In some populations, iron deficiency is estimated to reach 85% (Kapur *et al.*, 2002). In the United States, approximately 75% of college-aged women have low iron intake (Ramakrishnan *et al*., 2002), and suboptimal dietary intake of iron occurs in 90% of pregnant Americans (Swenson *et al.*, 2001).

The iron present in the human body is mostly in a stored form, and losses are usually minimal. However, dietary intake of iron is needed to replace the iron lost by passage of stool and urine, shedding of skin, and sweating. In fact, after exercising, a person can loose up to 1 mg of iron (Vellar, 1968), but on average, losses represent around 0.9 mg of iron per day for an adult male and 0.8 mg per day for an adult female (DeMaeyer *et al*., 1989). Infectious diseases such as intestinal parasites (Thurnham, 1997), as well as menstruation (Hallberg, 2001) can also increase iron requirements (due to elevated iron losses).

The intake of dietary iron is also linked to energy intake. In developed countries, a typical diet contains about 6 mg of iron per 1,000 kcal (equivalent to a daily consumption of 8 to 18 mg iron by most adults) with little variation from meal to meal or among persons of different economic status (Cook and Finch, 1979).

The Recommended Dietary Allowance (RDA) for a nutrient, as defined by the Institute of Medicine (1997), is "the average daily dietary nutrient

intake level sufficient to meet the nutrient requirement of nearly all (97 to 98 percent) healthy individuals in a particular life stage and gender group". Requirements vary between individuals and every nutrient has a distribution that is described by a median and a standard deviation (SD) for different age and sex groups (Renwick *et al.*, 2004). The dietary daily iron requirements in humans are summarized in Table 1-1. For children, iron requirements vary between 7 to 11 mg per day. Adult males require 8 mg per day, and women can require up 27 mg per day during pregnancy.

Table 1-1. Daily Recommended Dietary Allowances (RDA) (mg) according to life-stage, group and sex. Adapted from Institute of Medicine (2001).

Age	Infant, Child	Male	Female	Pregnancy	Lactation
7-12 month	11				
1-3 years	7				
4-8 years	10				
9-13 years		8	8		
14-18 years		11	15	27	10
19-50 years		8	18	27	9
>50 years		8	8		

Infants, children and adolescents require iron principally for their expanding red cell mass and growing body tissue, and therefore have higher requirements than adults. Moreover, they eat less food and are thus at greater risk of developing iron deficiency (Institute of Medicine, 2001). For women, the requirements increase dramatically during the menstrual period and during the second and third trimester of pregnancy. During pregnancy, additional iron is required for the fetus, the placenta, and the increased maternal blood volume. During lactation, because menstruation usually ceases, iron requirements decline.

2.2 Iron requirements in animals

Iron has been recognized as a required nutrient for animals for more than 100 years (Pond *et al.*, 1995). Still, sub-clinical iron deficiencies occur more frequently than recognized by most livestock producers. Currently, micronutrient deficiency is a bigger problem than macronutrient deficiency, because the farmer does not readily see specific symptoms that are characteristic of a trace mineral deficiency. Instead, the animal grows or reproduces at a reduced rate, uses feed less efficiently and operates with a depressed immune system (Berger, 2000). Both iron deficiency and iron excess can compromise the immune system of farm animals. Hypoferremia is believed to be an important protective component of the acute phase response to infection (Ebersole and Capelli, 2000). It is proposed that the

decrease in red blood cells, in the acute phase following infection and inflammatory disease, is a strategy to decrease iron availability to pathogens (Ebersole and Capelli, 2000). On the other hand, anemic animals are much more susceptible to infections than those with adequate iron. Nursing pigs made anemic by withholding supplemental iron for four weeks after birth were more susceptible to the lethal action of bacterial endotoxin then their littermates that had been given iron (Osborn and Davis, 1968). Once the infection was established, iron supplementation increased the bactericidal activity of liver and splenic macrophages. In another example, chicks inoculated with *Salmonella gallinarum* had increased survival when iron (100 µg/g of diet or more) was added to a basal diet containing 200 µg/g of iron (Hill *et al.*, 1977; Smith *et al.*, 1977). These and other data in broilers show that iron helps the immune system to destroy the invading organism. Therefore, proper iron nutrition is essential for maximal disease resistance.

Table 1-2. Dietary iron requirements (mg/kg of diet dry matter) in different animal classes (adapted from NRC 1981, NRC 1985, NRC 1989, NRC 1994, NRC 1996, NRC 1998, NRC 2001).

Class of Animal	Iron Requirements (mg/kg of diet dry matter)
Swine	
Piglets	100
Growing-finishing	40 – 80
Dairy cattle	
Calves	100
Other cattle	25
Beef cattle	50
Sheep	30 – 50
Goats	30 – 50
Horses	40 – 50
Poultry	50 – 80

Several factors influence the iron nutritional status and/or iron needs of animals. These include: 1) genetic differences amongst species, breeds, strains, stocks, sexes or individuals, 2) life cycle stage, with special emphasis on growth, pregnancy and lactation, 3) health status of the animal, 4) form of iron used in the diet (e.g. type of chelator), and 5) nutritional and anti-nutritional factors taken together with the iron source (NRC, 1989). Although there are minimally established nutrient needs for animals, designed to minimize the risk for deficiency, farm animals are usually fed to maximize their mass and/or to increase production in terms of meat, eggs, or milk (Grusak and Cakmak, 2005). Thus, mineral recommendations for production purposes are usually higher than those to prevent deficiency. Table 1-2 gives the feed iron recommendations for different classes of animals at different growth stages. When there is a range of requirements for

the same animal class, the higher value corresponds to the animal at a younger growth stage, or to females during pregnancy or lactation. The values for feed composition recommendations range from 25 mg/kg for adult dairy cattle up to 100 mg/kg for piglets or calves.

Table 1-3. Iron (mg) content of selected plant foods, raw, per common measure and per 100 g FW basis. Adapted from USDA National Nutrient Database for Standard Reference, Release 17, 2004.

Description	Common measure	Iron content (mg)	
		Per common measure	Per 100 g FW basis
Fruit			
Apple	1 apple	0.17	0.12
Avocado	1 oz	0.17	0.60
Banana	1 banana	0.31	0.26
Nectarine	1 nectarine	0.38	0.28
Strawberry	1 cup	0.70	0.42
Legumes			
Black Bean	1 cup, cooked	3.61	2.10
Chickpea	1 cup, cooked	4.74	2.89
Lentil	1 cup, cooked	6.59	3.33
Vegetables			
Cabbage	1 cup	0.41	0.58
Carrot	1 cup	0.33	0.30
Lettuce	1 cup	0.23	0.42
Potato	1 potato	2.18	1.10
Spinach	1 cup	0.81	2.70
Tomato	1 tomato	0.33	0.26
Nuts			
Almond	1 oz (24 nuts)	1.22	4.30
Cashew	1 oz (18 nuts)	1.72	6.06
Cereal Grains			
Barley	1 cup, cooked	2.09	1.33
White Rice	1 cup, cooked	0.24	0.14

3. PLANTS AS IRON SOURCES

3.1 Plants as iron sources for humans

Plant foods can contribute significantly to human nutrition and health because they contain almost all essential human nutrients (Grusak and Dellapena, 1999). Historically, humans have had an omnivorous diet. However, not all plant sources provide the same amount of iron, and the amount of iron ingested is directly proportional to the portion size that is consumed. Table 1-3 gives an example of the amount of iron (mg) in

selected raw plant sources, per 100 g fresh weight (FW) and per common serving size.

In the selected plant foods described, legumes, especially lentils, show substantially higher iron concentrations than fruit, cereal grains, and many vegetables. Based on 100 g FW, nuts such as almonds and cashews have very high iron content, whereas fruits generally have very low iron. As noted in Table 1-3, iron content varies among different plant foods, and nutrient content in a single serving rarely fulfills the RDA for any given vitamin or mineral.

Table 1-4. Iron concentration in different types of forages commonly used in the United States (adapted from Mortimer *et al.*, 1999).

Type of forage	Iron concentration (μg/g)	Sample #
Alfalfa / Alfalfa Mix	210 ± 13	196
Brome	156 ± 21	20
Bermuda	165 ± 37	120
Fescue	154 ± 22	73
Orchardgrass / Orchardgrass Mix	119 ± 15	34
Sudan	321 ± 41	61
Native grasses	179 ± 34	38
Cereal type forages (mixture of barley, oats and wheat)	174 ± 32	46
Silage / Silage grass (mixture of corn, sorghum and small grains)	252 ± 37	31
Grass (mixture of native and cultivated forages)	153 ± 18	70

Guthrie and Picciano (1995) found that the contributions of various food groups to the iron content of the North American diet were as follows: cereal products (43%); meat, fish, and poultry (22%); vegetables and beans (20%); eggs (3%); fruit (3%); and all other sources combined (9%). In a study conducted in China, it was found that grain products were the major food sources of iron (38%), with vegetables and legumes contributing 14 and 7% of dietary iron, respectively. The proportion of dietary iron acquired from meat, poultry and fish was only 13% (Liu *et al.*, 2004a). This tells us that at least 66% of the iron regularly consumed by North Americans and 59% for Chinese comes solely from plant sources.

3.2 Plants as iron sources for animals

Forage, either harvested mechanically or by grazing, is the basal dietary ingredient for beef cattle, dairy cattle, sheep, and horses. For poultry

production, the most common forages used are grains, such as corn, wheat, barley and oats. Other by-products commonly used are soybean, canola, cottonseed, and peanut. Mortimer *et al.* (1999) summarized feed analysis data from 709 forage samples, collected from 678 producers in 23 states in the US, and combined the data into 10 forage categories (Table 1-4). Values ranged from 119 μg/g in orchard grass to 321 μg/g in Sudan forage. These values were calculated from commercial forages and most likely represent samples that were contaminated with soil, dust, or other non-plant substances. Thus, the values do not necessarily reflect the plant's ability to acquire/store iron. However, these values do represent what the farmer is providing to his/her animals.

Even with some of these high levels in forage, anemia can still develop especially when an animal's total body iron stores are low. This often occurs in younger animals due to low iron content in their mother's milk (NRC, 1995). Because of this, iron supplementation is often provided during an animal's first weeks of life (Berger, 2000).

4. POSSIBLE SOLUTIONS TO INCREASE IRON STATUS IN PLANT SOURCES

We have seen that plants are essential sources of iron in the human and animal diet and that often iron concentration in plants is not enough to meet the daily dietary recommendations. In many parts of the developing world, due to elevated costs, large segments of the human population do not have access to animal sources of iron. In these cases, a commonly used strategy is iron fortification. However, iron fortification of plant foods is not always practical or economically feasible for the rural poor, and many times this fortified iron is not highly bioavailable (Boccio and Iyengar, 2003). Therefore, a more sustainable approach, that is believed relevant to both urban and rural populations, is to enhance the iron content of plant foods through biofortification. Biofortification is a process whereby the plant uses its own mechanisms to fortify or enhance the density or bioavailability of nutrients (like iron) in its edible tissues. To develop iron biofortified plants, four main strategies can be utilized: 1) cultivar evaluation, 2) plant breeding and marker-assisted selection, 3) alteration of pathways of iron metabolism, and 4) modification of iron bioavailability.

4.1 Cultivar evaluation

In the plant kingdom there is vast genetic variation that influences plant type, morphology, physiology, and plant mineral concentration. This

variation is visible at the species level, genotypic level, and even amongst individual plants. Moreover, developmental stages, stress, and environmental conditions also can influence iron concentrations. Rice genotypes, for instance, have been identified that vary from 6 to 24 µg/g iron in their grains (Gregorio *et al.*, 2000). Wheat can show a density range for iron concentration from 25 µg/g to a high of 56 µg/g, with a mean of 37 µg/g (Monasterio and Graham, 2000). Legume crops also show considerable variation in iron concentrations: wild accessions of common bean (*Phaseolus vulgaris*) can range from 60 to 95 µg/g iron in seeds, and cultivated bean ranges from 55 to 89 µg/g for seed iron (Beebe *et al.*, 2000). Table 1-5 shows how iron concentration in crops can vary not only amongst different species, but also in the same species grown at different locations. Plant breeders can utilize this genetic variation to generate or select varieties that have higher iron in the edible portions of the plant. These selections can be promoted directly as biofortified varieties, if other agronomic traits are acceptable. However, after having identified a potentially useful variety, it must be tested in multiple environments where the crop is to be grown, in order to assess whether the mineral trait will be stable in every condition.

4.2 Plant breeding and marker-assisted selection

Plant breeding has the potential to contribute to the development of cultivars with higher accumulation of nutrients, particularly iron. Until recently, however, plant breeders have focused primarily on increasing yield and improving disease resistance in crops, rather than improving micronutrient concentration in edible tissues (Frossard *et al.*, 2000). Iron concentration in plants is a quantitatively conditioned trait, showing continuous variation among individuals in a given population (Guzmán-Maldonado *et al.*, 2000). Therefore, breeding for high iron plants is a viable strategy, although sure to be a difficult one. With the identification of genotypes demonstrating a high iron seed phenotype, these can be moved into a conventional breeding program to combine the iron trait with other required characteristics. In general, a useful cultivar will need to demonstrate such things as high yield, good disease resistance, tolerance to environmental stress, and/or good processing qualities. The high iron phenotype alone will not be sufficient to warrant a cultivar release. The farmer must still be able to generate a high-yielding crop in order to make a profit, and unless people are willing to eat the final product, the biofortified line will provide no nutritional benefit to the target population. When breeding for higher levels of mineral nutrients, there is no direct visual score that can be used to screen progeny for the high-iron trait. Usually, in order to find individuals with a higher accumulation of a given mineral, the breeder must analyze plant

material using techniques such as ICP-AES (Inductively Coupled Plasma-Atomic Emission Spectrometry) or AAS (Atomic Absorption Spectrometry). For seed foods, this requires that all selections be grown to maturity, and that a portion of the generated seeds must be destructively analyzed. Although this approach will work, a more time- and resource-saving approach is to use Marker-Assisted Selection (MAS) techniques, following the identification of quantitative trait loci (QTLs) that are associated with high seed iron levels. QTL analysis and MAS have proven to be valuable tools in many breeding programs, especially in wheat and rice (Steele *et al.*, 2004), but practical applications of these techniques in micronutrient breeding have been limited.

Table 1-5. Ranges in iron concentration in various staple food crops grown in different locations: CIMMYT (International Maize and Wheat Improvement Center, Mexico); IRRI (International Rice Research Institute, Philippines) and CIAT (International Center for Tropical Agriculture, Colombia).

Plant Species	Number of genotypes	Iron (µg/g)		
		Min	Max	Mean
Wheat grown at CIMMYT (Monasterio and Graham, 2000)				
Selected genotypes	170	25	56	37
Pre-breeding lines	154	32	73	43
Rice genotypes grown at IRRI (Gregorio *et al.*, 2000)				
Traditional and improved	140	8	24	13
IR breeding	350	8	17	11
Tropical japonicas	250	9	24	13
Aromatic rice	51	11	23	15
Maize grown at CYMMYT (Bänzinger and Long, 2000)				
Landraces	416	18	59	26
Germplasm pools	100	10	17	13
Breeding germplasm	100	27	57	32
Bean grown at CIAT (Beebe *et al.*, 2000)				
Wild genotypes	119	-	96	60
Cultivated genotypes	1031	34	98	55
Cassava grown at CIAT (Chavez *et al.*, 2000)				
Leaves	20	62	155	94
Roots	20	8	13	10

In rice, three loci (located on chromosomes 7, 8 and 9) were identified that are associated with high iron concentration in the seeds (Gregorio *et al.*, 2000); these explained 19–30% of the variation in seed iron concentration. However, the lines with higher iron concentration were mostly low yielding,

and therefore breeding efforts have been started to combine the high nutrient trait with higher productivity. Similarly, in common bean, two putative QTLs associated with seed iron were localized in linkage groups II and III; these explained 25% of the phenotypic variance (Guzmán-Maldonado *et al.*, 2000). Efforts must now be made to verify all of these markers.

Related QTL efforts have been undertaken with *Arabidopsis thaliana*, where the existence of a fully sequenced genome could provide an ability to identify specific genes within a QTL, rather than just a nearby marker. The mineral concentration of *Arabidopsis* seeds has been analyzed in individual lines of a recombinant inbred population (Vreugdenhil *et al.*, 2004). QTLs were identified for several minerals (Ca, Fe, K, Mg, Mn, Na and Zn), which explained up to 78% of the variation for a specific mineral. Map positions for several of the QTLs was confirmed by analysis of near isogenic lines (NILs). For Fe, two QTLs were found on chromosomes 1 and 5; these regions co-localized with a few previously identified iron-associated genes (*ZIP10* and *NAS1*; Vreugdenhil *et al.*, 2004). However, more work is needed to confirm the role of these genes in seed iron accumulation, as opposed to whole-plant iron efficiency.

QTLs associated with other iron metabolic traits have been mapped, such as traits associated with resistance to ferrous iron toxicity in rice (Wan *et al.*, 2003; Shimizu *et al.*, 2004), or to iron deficiency chlorosis in soybean (Lin *et al.*, 2000). Theoretically, QTLs associated with iron toxicity or iron chlorosis resistance could also have an association with nutrient accumulation in seeds, but the link is not a direct one. Clearly, a plant must have sufficient internal levels of iron in vegetative tissues before this pool of iron can be partitioned to the developing seeds; however, because iron delivery to seeds also is regulated by phloem processes (Grusak, 1994), iron efficiency QTLs QTLs will presumably explain only a portion of the seed iron phenotype.

4.3 Alteration of pathways of iron metabolism

Plant sources of iron include both xylem-fed leafy vegetables and phloem-fed seeds. Increasing the iron concentration of either type of plant food will usually require increases in total iron input to the plant (Grusak and Dellapena, 1999). This can potentially be achieved through genetic manipulation, either by over-expressing endogenous genes or expressing novel transgenes associated with iron metabolism. Different processes can be targeted for genetic transformation, including: 1) root iron acquisition, 2) transport through the vascular tissues, and 3) storage in edible tissues. A complete and impressive array of genes involved in these processes has been isolated from plants, animals, and microbes (Table 1-6). Many of the initial plant iron-related genes were identified in *Arabidopsis* and in barley (Curie

and Briat, 2003). However, sequence homology has led to the identification of many more iron-related genes and homologues in other important plant species such as tomato, wheat and rice (Gross *et al.*, 2003; Koike *et al.*, 2004; Li *et al.*, 2004).

When choosing to alter a plant by targeting its iron acquisition system, it is imperative that one fully understands the processes pertinent to that plant. Higher plants utilize one of two strategies for iron acquisition (Marschner and Römheld, 1994). Strategy I involves an obligatory reduction of ferric iron prior to membrane influx of Fe^{2+}; this strategy is used by all dicotyledonous plants and the non-grass monocots. Strategy II (used by grasses) employs ferric chelators, called phytosiderophores, which are released by roots and chelate ferric iron in the rhizosphere (Curie and Briat, 2003). When plants of either strategy are challenged with Fe-deficiency stress, the processes associated with one or the other strategy is up-regulated in the plant's root system (Grusak and Dellapena, 1999).

Genes involved in Strategy I and II type processes have been cloned and transformed into different plant species in an effort to alter iron metabolism. In tobacco, the yeast *FRE1* and *FRE2* genes (responsible for iron reduction in yeast) have been expressed, resulting in an increase in root iron reductase activity (Samuelsen *et al.*, 1998). However, this increase was not proportional to the elevation in iron concentration in the leaves, suggesting that other factors determine the maximum amount of total mineral that can be absorbed. This can happen, for example, at the level of root absorption, in which case expression of an iron transporter such as IRT1 could result in higher iron uptake. It also is important to realize, however, that once the root cells absorb iron, this iron must be transported to the plant's aerial tissues via xylem transport. The movement of iron from root cortical cells to the apoplastic xylem pathway is thus a critical first step in this process (Stephan, 2002), and perhaps there may be a need to manipulate this transport step in order for the plant to "keep pace" with the increased influx of iron.

Once in the leaves, and prior to storage, iron appears to be reduced to Fe^{2+} before influx into leaf cells and various cellular compartments (Brüggemann *et al.*, 1993). A large portion of shoot iron is accumulated in chloroplasts, where it plays a strong role in the synthesis of chlorophyll. In chloroplasts, or other plastids, it usually is stored in the central core of the multimeric ferritin protein (Briat *et al.*, 1999). For leafy vegetables, improvement in iron concentration could be achieved by modifying ferritin levels. Tobacco transformed with a bean *ferritin* gene demonstrated a 3-fold increase in leaf iron concentration (Van Wuytswinkel *et al.*, 1998).

Table 1-6. Some of the major iron-related genes described in yeast and different plant species and their respective protein functions.

Gene	Reference	Protein function	Organism
CCC1	Li *et al*., 2001	Iron transport to the vacuole	*Saccharomyces cerevisiae*
FET1 – FET5	Dix *et al.*, 1994, Spizzo *et al*., 1997, Protchenko *et al*., 2001	Iron transport	*S. cerevisiae*
FRE1 – FRE7	Martins *et al*., 1998; Yun *et al*., 2001	Iron reduction	*S. cerevisiae*
FTR1	Stearman *et al*., 1996	Iron permease	*S. cerevisiae*
FRO2	Robinson *et al.*, 1999	Iron reductase	*Arabidopsis thaliana*
IRT1 – IRT2	Eide *et al*., 1996, Vert *et al*., 2002, Varotto *et al*., 2002	Root iron transporters	*A. thaliana*
NRAMP1, 3, 4	Curie *et al*., 2000; Thomine *et al*., 2000	Divalent metal transporters	*A. thaliana*
NaatA, NaatB	Takahashi *et al.*, 1999; Takahashi *et al*., 2001	Phytosiderophore biosynthesis	*Hordeum vulgare*
NAS	Higushi *et al*., 1999	Phytosiderophore biosynthesis	*H. vulgare*
IDS1 – IDS3	Nakanishi *et al*., 2000; Kobayashi *et al.,* 2001	Phytosiderophore biosynthesis	*H. vulgare*
MtZIP3, 5, 6	López-Millán *et al*., 2004	Iron transport	*Medicago truncatula*
FRO1	Waters *et al*., 2002	Iron reductase	*Pisum sativum*
ITP	Krueger *et al*., 2002	Peptide iron chelator	*Ricinnus communis*
YSL1 – YSL8	Basso *et al*., 1994, Curie *et al*., 2001	Phytosiderophore transport	*Zea mays*

Besides storage in leaves, iron also is transported to seeds and other terminal sinks. The peptides nicotianamine (found in all plants) and ITP (identified in *Ricinus communis*) are two putative candidates for iron transport in the phloem (Stephan and Sholtz, 1993; Krueger *et al.*, 2002; Schmidtke *et al.*, 1999). In fact, it has been speculated that nicotianamine may serve to shuttle iron to and from ITP during the loading and unloading of iron from the phloem (Curie and Briat, 2003); therefore, increasing the endogenous levels of these compounds could potentially result in higher iron

translocation to the seeds, assuming adequate iron substrate in the leaves. This could be especially important in cereal crops such as rice, whose seeds import only about 15% of total shoot iron (Grusak, unpublished). More information is needed, however, on the occurrence of ITP homologues in other species.

A possible strategy to accumulate iron in the seeds of plant species is to transform the plants with the *ferritin* gene driven by an endosperm specific promoter (Lucca *et al.*, 2002; Vasconcelos *et al.*, 2003; Liu *et al.*, 2004b). Manipulation of the *ferritin* gene and promoter sequences has proven effective in increasing the amount of iron accumulated in rice grains (Vasconcelos *et al.*, 2003; Liu *et al.*, 2004b). However, although seed iron levels were found to increase by as much as 64%, even higher levels are needed to approach the RDAs for iron, given the daily amounts of rice eaten by men, women, or children.

Finally, another important aspect of transgenic strategies will be the identification of novel *cis*-acting elements and promoters that can drive gene expression more efficiently. Recently, the IDE1 and IDE2 *cis*-acting elements of the *IDS2* gene from barley were isolated and found to confer iron deficiency-inducible gene expression (Kobayashi *et al.*, 2003). At the 12th International Symposium on Iron Nutrition and Interaction in Plants, held in Japan in 2004, several innovative strategies were described including construction of artificial promoters that are highly responsive to iron deficiency. Strategies of this sort should help the process of transformation by making it more directed and efficient.

4.4 Modification of iron bioavailability

Even if the concentration of iron in plants is increased, not all iron will be absorbed. Therefore, iron bioavailability also can be targeted, either by: 1) reducing or eliminating the presence of specific anti-nutritional factors, or 2) increasing or adding promotive compounds.

Phytate, a common compound in the seeds of many cereal grains, can lead to severe inhibition of iron absorption through its ability to complex ferric iron. To increase iron bioavailability, phytases can be introduced in order to degrade phytate, thereby liberating iron. Lucca *et al.* (2002) introduced a phytase from *Aspergillus fumigatus* into rice endosperm in an effort to enhance iron bioavailability. However, once expressed in the plant, this phytase was not found to be heat stable. More work is needed to identify a thermo-tolerant phytase that can resist the high temperatures of cooking. Alternatively, low seed-phytate mutants of some crop species have been identified (Raboy, 2001), and human nutritional studies have shown an improvement in zinc bioavailability when subjects were fed a low-phytate

maize mutant (Hambidge *et al.*, 2004). Presumably, iron bioavailability would be equally enhanced.

Tannic acid is a complex polyphenolic that can inhibit iron absorption because of its galloyl-containing group, which binds iron and thus inhibits its absorption from food (Afsana *et al.*, 2003; Afsana *et al.*, 2004). Tannins exist in both gymnosperms and angiosperm species and have a very widespread distribution, being found in buds, leaves, roots and seeds (Reed, 1995). Reduction of tannin levels in seed coats of bean is possible through breeding (Guzmán-Maldonado *et al.*, 2000), and the elimination of tannins from vegetative tissues (e.g. in forage species) could be possible through genetic engineering (Dixon *et al*., 2005). However, the removal of tannins would have some potential drawbacks for the plant, in that tannins can provide protection against freezing, predators, and microbial pathogens (Reed, 1995). As an alternative, it could be possible to transform the plant in order to produce compounds that will prevent the anti-nutritional properties of tannins. Recent studies have shown that difructose anhydride III (DFA III), an indigestible saccharide, can partially prevent the tannic acid-induced suppression of iron absorption, at least in rats (Afsana *et al.*, 2003). DFA III can be synthesized from inulin by an inulin fructotransferase (Inulinase II) (Ushyama, 1975). Inulin is also a non-digestible dissacharide compound, and it is found in some plants. DFA III is not hydrolyzed by enzymes in the small intestine, but is metabolized by microorganisms in the large intestine. The activity of this compound should remain active even after cooking, and fortunately, evidence shows that DFA III is very stable at high temperatures, and at acidic conditions (pH 2.0 at 100°C for 30 min) (Afsana *et al.*, 2003).

Other compounds reported to have a beneficial effect on iron absorption are water-soluble soybean fiber (WSSF) (Shiga *et al.*, 2003), short-chain fructooligosaccharides (Sakai *et al.*, 2000), and 1-25 caseinophosphopeptide (beta CPP), which is obtained from the hydrolysis of beta casein (Ait-Oukhatar *et al.*, 1999). These compounds have been shown to prevent iron-deficiency anemia in rats by stimulating iron absorption in the small and large intestine. Ascorbic acid (ascorbate) also can enhance iron bio-availability (Walter *et al.*, 2004; Yun *et al.*, 2004), presumably through its ability to reduce ferric iron. Recent gene discovery in the ascorbate biosynthetic pathway (Jain and Nessler, 2000) could be leveraged to increase levels of this compound in seeds of different species. Finally, amino acids such as cysteine can act as enhancers of iron absorption (Layrisse *et al.*, 1984). Lucca *et al.* (2002) over-expressed an endogenous cysteine-rich metallothionein-like protein in transgenic rice carrying the *ferritin* gene and were able to increase the content of cysteine residues sevenfold. This strategy could be attempted in other species.

5. CONCLUSIONS

It is clear that plants play an important role as dietary sources of iron for humans and other animals. However, recommended dietary intakes of iron are not always met. Iron concentrations in some common foods are low and iron bioavailability can be poor. Thus, there is a need to enhance the iron nutritional value of edible plant products in order to improve the food supply. Efforts are underway to screen germplasm for genotypes with elevated seed iron concentrations, and quantitative loci for iron-related traits are beginning to be identified. These efforts, along with the growing body of knowledge concerning iron-related genes, should enable the sensible development of marker-assisted breeding strategies and/or transgenic approaches that will yield nutritionally improved cultivars in the coming years Additionally our expanding knowledge base in the area of iron bio-availability can provide opportunities for manipulating non-iron food components that also will benefit the iron nutritional quality of the edible tissues.

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Chapter 2

IRON NUTRITION IN FIELD CROPS

NEIL C. HANSEN[1], BRYAN G. HOPKINS[2], JASON W. ELLSWORTH[3] and VON D. JOLLEY[4]

[1]Department of Soil and Crop Sciences, 1170 Campus Delivery, Colorado State University, Fort Collins, Colorado 80523-1170, USA. [2]Cropping Systems, 1776 Science Center Dr., University of Idaho, Idaho Falls, ID, USA. [3]Twin Falls R & E Center, University of Idaho, 315 Falls Avenue, Evergreen Building, P.O. Box 1827, Twin Falls, Idaho 83303-1827, USA. [4]Department of Plant and Animal Sciences, Brigham Young University, 253 WIDB, Provo, UT 84602, USA.

Abstract: Iron (Fe) deficiency is a yield-limiting factor for a variety of field crops across the world and generally results from the interaction of limited soil Fe bioavailability and susceptible genotype cultivation. Iron deficiency broadly occurs across the world in soybean, peanut, dry bean, sorghum, and rice but sporadically under unique or specialized conditions with corn, wheat, and oat. In this chapter, soil properties associated with the expression of Fe deficiency in field crops are defined, and biochemical interactions that promote field observed Fe-deficiency problems are explored. Strategies examined for use with field crops where Fe deficiency is a concern include cultivar selection and screening for tolerance, fertilizer and cultural practices, lowering soil pH, foliar sprays, chelated and complexed Fe fertilizers, concentrated fertilizers, mixed cultivar and companion crop interactions, and management of irrigation and drainage, fertility and seeding practices. Finally, we identify areas where future research is needed.

Key words: cultivar screening; geographic distribution; interactions; management; soil properties

1. INTRODUCTION

Iron (Fe) deficiency is a yield-limiting factor with major implications for field crop production in many agricultural regions of the world. It is generally a result of a combination of limited bioavailability of soil Fe (even though total soil Fe content may be relatively high) and culture of

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susceptible genotypes that have insufficient activity of one or more Fe-deficiency response mechanisms (Brown and Jolley, 1989). In addition, there are notable biological interactions with Fe-stress response mechanisms that can result in Fe deficiency, even for Fe-efficient plant types. Less common are Fe deficiencies for crops grown on soils low in total Fe content, although this can occur on some sandy or organic soils. In this chapter, we provide several examples where Fe deficiency is a significant production issue for field crops in different geographic regions. We review soil properties associated with the expression of Fe deficiency in field crops and explain interactions that cause some field observed Fe deficiency problems. Finally, we outline key themes for the management of field crops where Fe deficiency is a concern and identify areas where future research is needed.

2. OCCURRENCE OF IRON DEFICIENCY IN FIELD CROPS

Iron deficiencies have been reported for many plant species and geographical regions. The most common problems correspond to the cultivation of sensitive crop species (Table 2-1) in arid and semi-arid regions with calcareous soils (presence of free carbonates). Iron deficiency is widely reported in soybean [*Glycine max* (L.) Merr.], peanut (*Arachis hypogaea* L.), dry bean (*Phaseolus vulgaris* L.), sorghum [*Sorghum bicolor* (L.) Moench], and rice (*Oryza sativa* L.). Additional Fe deficiency is reported under unique or specialized conditions with corn (*Zea mays* L.), wheat [*Triticum aestivum* (L.).Thell.], oat (*Avenae sativa* L.), and other minor crops.

Table 2-1. The relative sensitivity or tolerance of crops to low levels of available Fe in soil. Some crops are listed under more than one category because of variations in the response of different varieties of a given crop. Modified from Tisdale *et al.* (1985).

Sensitive	Moderately Sensitive	Tolerant
Field bean	Alfalfa	Barley
Forage sorghum	Corn	Cotton
Grain sorghum	Cotton	Millet
Peanut	Field pea	Oat
Soybean	Forage legume	Potato
Sudangrass	Rice	Sugar Beet
	Soybean	Wheat

One of the most widely studied species suffering Fe deficiency chlorosis among field crops is soybean. Iron deficiency in soybean has been a significant yield-limiting factor for decades. Despite extensive study, the incidence of Fe deficiency is increasing primarily due to expansion of soybean production into areas where soil conditions that exacerbate

deficiency prevail. The acreage of soybean affected by Fe deficiency has been estimated to have expanded by 160% in the last three decades, potentially affecting nearly two million hectares and reducing yields an average of 0.75 Mg ha^{-1} (Figures 2-1 and 2-2; Hansen *et al.*, 2004). The decades of research with Fe deficiency in soybean have elucidated an understanding of Fe deficiency stress response mechanisms (Jolley and Brown, 1994) and identified a wide variation among soybean varieties in susceptibility to Fe deficiency. A strong correlation exists between the susceptibility of varieties in the field to Fe deficiency and the degree to which these varieties express Fe-deficiency response mechanisms under controlled conditions (Jolley *et al.*, 1992; Stevens *et al.*, 1993). Private companies and public institutions routinely rank soybean varieties for susceptibility to Fe deficiency. Despite the extensive research and variety screening efforts, Fe deficiency continues to be a challenge in soybean production (Franzen and Richardson, 2000; Goos and Johnson, 2000; Hansen *et al.*, 2003; Inskeep and Bloom, 1984).

Figure 2-1. Geographical distribution of Fe deficiency in field crops in the U.S. Modified after Berger and Pratt (1963).

Peanut is another dicotyledenous species sensitive to Fe deficiency and there are production challenges associated with Fe deficiency in various regions (Frankel *et al.*, 2004). Peanut cultivated in the southern Great Plains (Reed and Ocumpaugh, 1991) of the U.S., northern China (Zuo *et al.*, 2004) and Israel (Hartzook *et al.*, 1974; Hartzook, 1984) often develops Fe deficiency. The problem is particularly prominent with the presence of a caliche layer near the soil surface, which can induce perched water

conditions in surface soils. When grown as a monocrop on calcareous soils, Fe deficiency commonly limits peanut yield in Northern China production areas (Zuo *et al.*, 2004), while the problem is rare when peanut is intercropped with corn. This difference is attributed to a complex of interactions that improve Fe uptake and nitrogen (N_2) fixation (Zuo *et al.*, 2004).

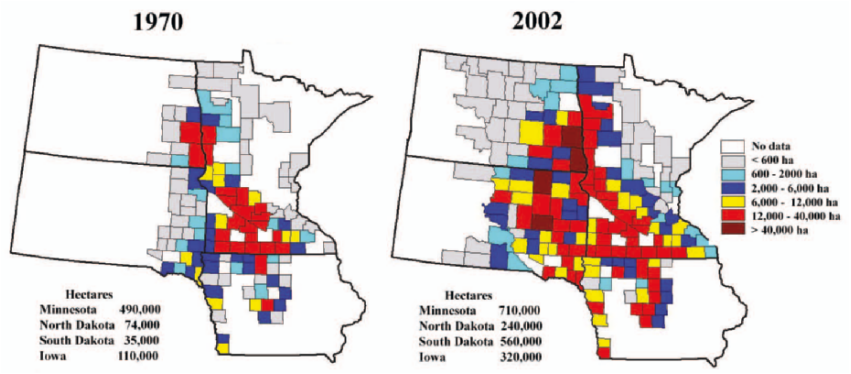

Figure 2-2. These maps of the North Central U.S show the states of North Dakota, South Dakota, Iowa and Minnesota. The maps show the land area in each county where soybean was planted on soils with pH >7.2 in 1970 and 2002. A 160% increase in soybean production on high pH soils between 1970 and 2002 is partially responsible for an increase in the importance of iron deficiency chlorosis in soybean in this region.

Iron deficiency is also a problem in dry bean. Dry bean most often suffers Fe deficiency in the same soil conditions as soybean. Research in the central U.S. (Nuland *et al.*, 1997) shows differing responses to high pH soils among 24 dry bean cultivars. The most tolerant cultivars remained green all season with normal yields and the least resistant cultivars suffered extensive yield loss. These observations were confirmed by Ellsworth *et al.* (1997, 1998) when they measured the physiological response to Fe deficiencies. Their results correlated with field scores ($r = -0.71$ to -0.49). In Tunisia, Krouma *et al.* (2003) observed differences in physiological responses and chlorosis development in five common bean cultivars, but did not relate the differences to field conditions. These studies indicate that some varieties are more tolerant of conditions that make Fe less available and effort needs to be placed in management and choice of the cultivar rather than in-season fertilization.

Another widely impacted crop is sorghum. Monocots, such as sorghum, release naturally occurring chelates (phytosiderophores) in response to Fe deficiency (Clark *et al.*, 1988; Lytle and Jolley, 1991), but the magnitude of phytosiderophore release is minor compared to more tolerant crops, such as

corn and wheat (Onyezili and Ross, 1993). Thus, grain sorghum is particularly sensitive to Fe deficiency problems in soils with low available Fe. Significant areas of Fe deficiency have been reported for sorghum in the Southern Great Plains region of North America including large areas in the states of Texas and Oklahoma. Iron-deficiency symptoms in sorghum are typically expressed in young plants, but become less apparent as the plant matures. The visual symptoms of Fe deficiency are spatially variable in fields, often associated with calcareous outcroppings. In addition to yield losses, sorghum that is deficient in Fe at early growth stages experiences other production challenges such as uneven flowering and delayed grain ripening (Livingston *et al.*, 1996). In severe cases, Fe deficiency can cause a total crop failure.

Rice is moderately sensitive to Fe deficiency, but the problem is relatively rare (Dobermann and Fairhurst, 2000). When rice is produced in flooded conditions, soil Fe is reduced to the more soluble Fe^{+2} forms, even in calcareous soils. Zinc (Zn) deficiency is a more common problem in rice production, while in some cases there may be simultaneous deficiencies in both Zn and Fe. There are occurrences of Fe deficiency in rice in the U.S. under certain conditions: inadequate soil reduction due to low organic matter levels, calcareous subsoil conditions, and large ratios of phosphorus (P) to Fe, where low solubility iron phosphates form (Morikawa *et al.*, 2004). Iron deficiency occurs in the Texas and Louisiana rice production areas in high pH, sandy soils with low soil Fe levels (Fred Turner, Texas A&M, Beaumont, TX, personal communication) and in areas of the Florida Everglades region on peat soils with low total Fe content where Fe fertilizer must be applied (Snyder and Jones, 1988). Iron deficiencies have also been observed in areas of rice production in Indo Gangetic Plains area of Pakistan and India. The common rice production system in this region is a rice wheat rotation, with rice culture being done by transplant (Agrawal and Srivastava, 1984; Singh *et al.*, 2004; Timsina and Connor, 2001). Iron deficiency occurs in early transplants and depends highly on irrigation and flooding practices. Yields can be dramatically reduced, even though plants regreen as they mature. Alternatively, Fe deficiency is rare in the Arkansas and California rice production areas.

Corn is only moderately sensitive to Fe deficiency problems (Table 2-1). However, Fe deficiency of corn has been reported as a production problem in some areas of the central Great Plains and Western U.S., but is generally not a problem in the majority of the corn growing areas. However, Fe deficiency in corn is observed in the flood plains of the river valleys of Nebraska on high pH and sodium affected soils and reduces corn yields on as many as 500,000 ha (Nordquist *et al.*, 1992) and is reported to occur on millions of hectares on a global scale (Nordquist *et al.*, 1996). The

occurrence of Fe deficiency in sodic soils suggests that the Fe uptake mechanisms are impaired as a result of root stress from soil salts. Plant selection efforts have identified moderately productive corn hybrids for these soils, but have not been able to completely eliminate yield losses associated with Fe deficiency (Nordquist *et al.*, 1992, 1996). Due to the heterogeneous nature of Fe deficiency, one production concern is whether to select the highest yielding hybrids even if they are sensitive to Fe deficiency, or to select moderately yielding hybrids that are tolerant to Fe deficiency.

Wheat and oat are tolerant to Fe deficiency, even when grown in soils where Fe deficiency may be a problem with other crops (Table 2-1). However, both oat and wheat show significant susceptibility to Fe deficiency under animal grazing practices, a major management scheme used on much of the wheat acreage in Southern Plains region of the U.S and in the oat acreage in South Texas. Grazing semi-dwarf winter wheat during the vegetative stage is common in the Southern Plains region of the U.S. This practice can reduce grain yields, but allows for economic gains through its use as forage (Winter and Thompson, 1990). Factors such as reduced leaf area and seed weight and increased winterkill contribute to lower grain yield in grazing systems, but in some instances, the grazing has also triggered Fe-deficiency chlorosis in the vegetative regrowth for wheat cultivars that do not normally exhibit chlorosis when not grazed (Berg *et al.*, 1993). Most of the oat grown in south Texas is grazed and grown in semiarid areas where soils are calcareous and Fe-deficiency chlorosis is a potential problem (Anderson, 1982). Grazing or clipping is known to intensify Fe chlorosis in oat in these areas as chlorosis is often enhanced following clipping in oat forage tests (Ocumpaugh *et al.*, 1992). Differential susceptibility to Fe-deficiency chlorosis among wheat and oat cultivars likely contribute to some of the unexplained cultivar x grazing interactions reported in yields of grazed wheat and oat (Winter and Thompson, 1990; Ocumpaugh *et al.*, 1992). In hydroponic studies it was shown that the ability to release adequate phytosiderophore following grazing is critical to Fe-deficiency chlorosis resistance in winter wheat (Hansen *et al.*, 1995).

3. SOIL PROPERTIES ASSOCIATED WITH IRON DEFICIENCY

Iron comprises approximately 5% of the earth's crust and is the fourth most abundant element in the lithosphere (Tisdale *et al.*, 1993), and as a result, plants are growing in a "sea of Fe". However, the bioavailability of Fe in alkaline soil is very low (Chen and Barak, 1982; Jolley and Brown, 1994; Lindsay and Schwab, 1982; Vose, 1982).

It is well documented that Fe deficiency in field crops primarily occurs in high pH, alkaline soils (calcareous conditions) (Hansen *et al.*, 2003; Inskeep and Bloom, 1984). These soils have pH in the range of 7.5 to 8.5, with higher pH values in sodium accumulating soils. The solubility of Fe minerals decreases exponentially for each pH unit increase in the pH range common for soil (Lindsay, 1974; Lindsay and Schwab, 1982). Free soil carbonate exacerbates the unavailability of Fe due to the formation of poorly soluble Fe phosphates. As much as one-third of the world's surface soils are calcareous, primarily in arid and semi-arid regions (Brown, 1961; FAO, 2005) with additional land underlain with calcareous subsoils. Although Fe deficiency is relatively common, most field crops have adapted to grow normally in these environments.

Iron deficiency generally occurs when susceptible crop varieties are cultivated in soils that promote the problem. In areas where Fe deficiency is a concern, the deficiencies generally occur heterogeneously rather than uniformly across a field. Chlorotic patches often occur in fields spatially uniform with respect to high soil pH, indicating that alkalinity is not the only factor controlling the availability and uptake of Fe (Hansen *et al.*, 2004). Other soil factors associated with the expression of Fe deficiency include: carbonates, salinity, Fe composition, moisture, bulk density, and concentration and form of interacting elements and compounds. In addition, environmental conditions interact to promote Fe deficiency problems. In some years severe Fe deficiency is observed, while in other years little or no Fe deficiency occurs in the same locations. Cool, wet conditions often aggravate Fe deficiency, but the effects of temperature and moisture are complex and often contradictory.

High levels of bicarbonates (HCO_3^-) in the soil solution will induce Fe deficiency stress (Coulombe *et al.*, 1984, Inskeep and Bloom, 1984). The concentration of HCO_3^- in the soil solution is affected by the concentration and reactivity of soil carbonates, exchangeable bases, soil moisture content, and the concentration of carbon dioxide (CO_2). Several studies have compared soil conditions where no or serious deficiency symptom is apparent in the same field. Some have documented that higher solid phase carbonate concentration measured as calcium carbonate equivalent (CCE) relates to chlorosis expression (Franzen and Richardson, 2000; Inskeep and Bloom, 1987). Others have found that soil CCE did not differ with different degrees of chlorosis, but that the reactivity of carbonates (Morris *et al.*, 1990) or the clay-sized fraction of carbonates had a strong relationship with Fe chlorosis (Inskeep and Bloom, 1986).

Soil salinity has also been linked to the occurrence of Fe deficiency in some crops, including soybean and corn. Reason for this relationship is lacking, but it is believed that decreased root growth with increased soil

salinity may be at least part of the cause. Inskeep and Bloom (1984) compared soil characteristics for soybean differing in Fe deficiency and found that low chlorophyll content was associated with higher soil Mg^{2+}, Na^{+}, and Cl^{-} concentrations and also a higher Mg/Ca ratio. Soluble salt concentration (EC) is related to differences in Fe deficiency chlorosis in soybean with higher EC observed for the chlorotic areas than for the non-chlorotic areas of the same field (Hansen *et al.*, 2004). Similar observations have been made elsewhere (Franzen and Richardson, 2000; Inskeep and Bloom, 1987; Loeppert *et al.*, 1994; Morris *et al.*, 1990).

Neither total nor extractable soil Fe concentrations are useful as predictors of the risk of Fe deficiency in field crops. Total Fe levels may be quite high at the same time that available Fe concentration is limiting due to low solubility (Lindsay and Schwab, 1982). More important than total soil Fe are the concentration, mineralogy, and crystalinity of soil Fe oxide (Vempati and Loeppert, 1988). The DTPA extractable Fe test has been used as an indicator of available Fe (Olson and Ellis, 1982). However, the identification of soils prone to Fe deficiency using soil DTPA-Fe has been mixed. Hansen *et al.* (2003) found that soil DTPA-Fe levels were lower in Minnesota production areas where soybeans showed Fe deficiency symptoms than in areas without symptoms, while Inskeep and Bloom (1987) concluded that there was no difference in soil DTPA-Fe levels when comparing chlorotic and non-chlorotic areas of individual fields in Minnesota. In North Dakota, Franzen and Richardson (2000) found differences in DTPA-Fe between chlorotic and non-chlorotic areas for some sites but not for others. McKeague and Day (1966) and Morris *et al.* (1990) found a positive correlation between leaf chlorophyll concentration and the quantity of amorphous Fe oxide in calcareous soils.

Soil moisture content relates to the expression of Fe deficiency with deficiency most likely to occur in wet, but unsaturated soils. In greenhouse trials, chlorosis expression is exacerbated for soybean and oat with increasing soil moisture in calcareous soils (Inskeep and Bloom, 1986; Ocumpaugh *et al.*, 1992). In field production of soybean, soil moisture was greater in areas where soybeans were Fe deficient than in areas where soybeans grew normally (Hansen *et al.*, 2003). A combination of the increase in soil bicarbonate concentration due to high soil-water content and associated poor soil aeration leading to oxygen deficit and the resulting root growth reduction is the possible cause of chlorosis. The differences in Fe chlorosis *versus* chlorosis from hypoxia are distinct enough to diagnose the problem. High soil moisture content also causes accumulations of volatile compounds, such as ethylene, in the root environment, which may affect root growth and induce chlorosis expression (Inskeep and Bloom, 1986).

Field observations indicate that chlorosis is affected by soil compaction and the associated change in soil bulk density. In some instances, chlorosis in tractor-wheel tracks is worse than chlorosis outside of wheel tracks. This may be related to the fact that as air-filled porosity of soil decreases due to compaction, there is an inhibition of diffusion of plant and microbial-produced CO_2 away from the rhizosphere. This localized accumulation of CO_2 results in an increase in concentration of HCO_3^- and a decrease of Fe availability (Geiger and Loeppert, 1988). In other instances, chlorosis in wheel tracks is less severe than in surrounding areas (Hansen *et al.*, 2004). This phenomenon has not been explained, but is likely related to impacts on soil temperature or water movement.

4. BIOCHEMICAL INTERACTIONS WITH IRON DEFICIENCY

One reason for the limited success in managing Fe deficiency in field crops is that the Fe deficiency may be a result of multiple soil or biochemical stresses and not simply to limited available Fe. In some cases, biological interactions with the Fe-stress response mechanisms (Brown, 1961; Brown and Jolley, 1989), can result in Fe deficiency, even for Fe-efficient plant types. Several examples of interactions that promote Fe deficiency in various field crops will be discussed.

4.1 Potassium

Insufficient K led to Fe deficiency in the field in potato (*Solanum tuberosum* L.), fruit trees, peanut, sugar cane (*Saccharum officinarum* L.), and corn, which indicates K is somehow involved in increased uptake and transport of Fe in plants (Hughes *et al.*, 1992). Inadequate K reduces or eliminates a plant's ability to express the normal Fe deficiency responses observed with adequate K nutrition (Hughes *et al.*, 1990; Jolley and Brown, 1985; Jolley *et al.*, 1988; Miller *et al.*, 1990; Szlek *et al.*, 1990). For example, the combination of Fe and K deficiency in tomato (*Lycopersicon esculentum* Mill.) and soybean decreased the release of hydrogen ions and Fe reducing compounds, as well as reduction of Fe^{3+} at the roots when compared to Fe deficient plants with adequate K (Hughes *et al.*, 1992; Jolley *et al.*, 1988). Similarly, muskmelon (*Cucumis melo* L.) roots did not reduce Fe nor release reductants, but did release hydrogen ions at a later time under the combination of Fe and K stress (Hughes *et al.*, 1990). Potassium could be involved in the function of two critical enzymes associated with Fe-stress response activity, ATPase and Fe^{3+} reductase, in H^+ ion symport, and

with the accumulation of citrate for Fe transport (Marschner *et al.*, 1986a). Potassium-activated, membrane-bound proton-pumping ATPases are common in higher plants. Furthermore, a stimulation of root plasma membrane ATPase activity by K has been reported (Mengel and Schubert, 1985). Impact of K on Fe^{3+} reductase activity may be indirect. Tagliavini and Rombolà (2001) hypothesized that plasmalemma ATPase activity and regulation may provide favorable apoplastic pH levels and improve Fe^{3+} reductase activity. Potassium could potentially affect Fe uptake *via* its general role in cation/ anion balance and in facilitating movement of organic acids such as citrate (Marschner, 1995).

Potassium has also been shown to interact with Fe nutrition in monocots. Significantly more phytosiderophore was released by the roots of an Fe-efficient cultivar of oat grown with sufficient K and subjected to Fe stress than by roots of that same cultivar grown with low K and Fe (Hughes *et al.*, 1992). The diminished release of phytosiderophores corresponded with increased Fe-deficiency chlorosis and lower leaf Fe concentration. They concluded that K plays a role in the production and release of phyto-siderophores. Potassium has multiple impacts in methionine biosynthesis, and methionine is a dominant precursor of phytosiderophore (Ma *et al.*, 1995; Mori and Nishizawa, 1987). In addition, work by Sakaguchi *et al.* (1999) showed that elimination of the K gradient between the cytoplasm and the cell exterior reduced the amount of secreted mugineic acid family phytosiderophores (MAs) and that K in the cortex cells of roots under Fe-deficiency stress is released with MAs secretion, decreasing the amount of K in the cortex cells. This suggests that MAs are secreted *via* channels using the K gradient between the cytoplasm and the cell exterior (Neumann and Römheld, 2001). Jolley and Bernards (Brigham Young University, Provo, UT, unpublished data, 2003) observed high levels of K in root exudates during the phytosiderophore collection period in Fe-deficiency stressed corn.

4.2 Sulfur

The dominant amino acid precursor of phytosiderophores in the Strategy II response is methionine, a S-containing amino acid (Mori and Nishizawa, 1987). Astolfi *et al.* (2003) grew maize plants with pretreatments of with and without sulfate and transferred half of each treatment to either Fe deficient or Fe sufficient solutions. Leaf Fe concentration was lower in the leaves of plants grown with S deficiency than in those grown with sufficient S. Astolfi *et al.* (2004) reported that inadequate S reduced phytosiderophore release from barley by 25 to 75% compared to adequate S and, as a result, uptake of Fe was decreased about 30%. Although yet to be confirmed in the field,

laboratory research shows that S deficiency leads to greater Fe-deficiency chlorosis because of its influence on phytosiderophore production.

Sulfur deficiency could limit the Fe stress response mechanisms of Strategy I plants, but the relationship has not been proven. Release of reductants from roots of Fe-stressed dicots in concert with other mechanisms enhances Fe^{3+} reduction in the soil solution. A limited number of species release riboflavin reductant compounds when under Fe stress. These are S-rich compounds. Susín *et al.* (1993) found that riboflavin 3'-sulfate and riboflavin 5'-sulfate were 97% of total riboflavin in Fe-stressed sugar beet (*Beta vulgaris* L.) roots. Location of riboflavin matched that of high Fe^{3+} reductase activity and quantities were adequate for them to hypothesize that these riboflavin sulfates could be important to the Fe-reduction system in sugar beet. Thus, S deficiency could potentially reduce Fe uptake in species producing riboflavin sulfates. However, the physiological significance of reductants has been frequently discounted based on a comparison of the amount of Fe reduced to the total Fe required by plants (Barrett-Lennard *et al.*, 1983).

4.3 Phosphorus

Excessive application of P fertilizers is known to cause economic losses in many crop species (Brown and Tiffin, 1962; Haleem *et al.*, 1992; Kadar, and Elek, 1999; Marschner, 1986a, 1986b, 1986c; Moraghan and Mascagni, 1991; Singh *et al.*, 1996). Brown and Tiffin (1962) found that Fe interacts with P in maize. Safaya (1976) established a linkage with the P-Zn interaction and Mn, Fe, and Cu uptake by maize. The causes of the reduction in crop yield and quality due to excess P have not been fully elucidated. However, it is likely that the primary reason is due to an antagonistic interaction with the cationic micronutrients, namely: Fe, Zn, Mn, and Cu. These nutrients are taken up by plants as cations and P is taken up by plants as orthophosphate anions. Positively and negatively charged ions have an electrical attraction to one another, facilitating the formation of a chemical bond that can form in either the soil or the plant tissue. The relative strength of the P micronutrient cation bond is strong and does not readily break without dramatic changes in the physical or chemical environment. If excess P binds a large amount of Fe normally used by the plant, the result can be a P-induced Fe deficiency. Conversely, excessive concentrations of one or more of the cationic micronutrients can result in reduced P uptake by plants (Pin and FuSuo, 1995; Torun *et al.*, 2001; Wallace, 1984; Wallace and Cha, 1989) or a deficiency of one of the other cationic micronutrients (Loneragan and Webb, 1993; Marschner, 1986a; Moraghan and Mascagni, 1991; Safaya, 1976, Safaya and Gupta, 1979; Wallace, 1984; Wallace and Cha, 1989).

4.4 Micronutrients

There are literally hundreds of known interactions among micronutrients, but a comprehensive review is not feasible in this paper. Rather, a few examples to illustrate the importance of micronutrient interactions with respect to the Fe-deficiency-stress response mechanisms will be discussed.

One of the most clearly defined interactions between Fe-stress response mechanisms and another micronutrient is an interaction with Zn in navy bean. The Zn-inefficient variety, Sanilac, readily exhibits Zn deficiency while the Zn-efficient variety, Saginaw, does not. It was also observed that Sanilac accumulates Fe under Zn-deficiency stress while Saginaw does not (Ellis, 1965). These two cultivars were grown hydroponically under Zn-deficient and Zn-sufficient conditions. Two of the three Fe-stress response mechanisms (reductant release and reduction of Fe^{3+} to Fe^{2+} at the root) were activated by Zn-deficiency stress in Sanilac (Jolley and Brown, 1991), but not in Saginaw. Consequently, Sanilac showed increased Fe uptake and aggravation of Zn-deficiency under Zn stress, while Saginaw prevents an imbalance of Zn and Fe by minimizing Fe uptake by actually reducing Fe reduction activity during Zn-deficiency stress.

The uptake of Fe is found to be closely related to the uptake of other micronutrients in Strategy I plants. Kobayashi *et al.* (2003) found that tobacco (*Nicotiana tabacum* L.) plants deficient only in Fe had more severe chlorosis than plants with a combination of two deficiencies i.e. Zn and Fe or Mn and Fe. The lone Fe deficiency stimulated an accumulation of Mn, Zn, and Cu in shoots, while combined Zn and Mn deficiency stimulated Fe accumulation in the shoots. However, the cause of this interaction does not appear to be due to the effects of Mn and Zn on the Fe (III) reductase enzyme. While the metals Ni, Cu, and Cd were shown to severely decrease the activity and function of the Fe (III) reductase enzyme, Mn, Pb, Zn and Mo had no effect on the Fe (III) reductase in Strategy I plants (Alcántara *et al.*, 1994). In a study regarding Cd uptake in Fe deficient pea (*Pisum sativum* L.) seedlings, Cohen *et al.* (1998) found the rate of Cd uptake was seven times faster in Fe-deficient seedlings and the increased uptake was not due to H^+-ATPase activity. Possible causes for this interaction include a chelating competition between the nutrients, competition for any molecule that transports or holds Fe such as citrate, malate, nicotianamine, mugineic acids, and Fe transport proteins; or the effects of these nutrients on a messenger molecule that determines the physiologic response of the plant (Kobayashi *et al.*, 2003). The Fe transporter, IRT1, transports several divalent metals in *Arabidopsis* including Fe, Cd, and Zn (Cohen *et al.*, 1998).

4.5 Dinitrogen (N_2) fixation

Wallace (1982) observed regreening of Fe chlorosis in nodulated soybean in greenhouse pots and Hansen *et al.* (2003) observed that Fe chlorotic soybeans in the field were often lacking nodule development. Symbiotic N fixation in *Bradyrhizobium*-infected legumes results in H^+ release into the rhizosphere and a reducing environment in the root nodule (Gibson, 1980). Both Fe-stress response and symbiotic N_2 fixation are biochemical reduction processes that occur in the root and which interact to enhance Fe-stress responses in soybean (Soerensen *et al.*, 1988; Terry *et al.*, 1991) and peanut (Terry *et al.*, 1988). For example, T203 soybean normally fails to produce any Fe-stress response. However, in the absence of both N and Fe, T203 roots inoculated with *Bradyrhizobium japonicum* responded to Fe-deficiency stress by exuding both H^+ and reductants and reducing Fe^{3+}. Iron reduction activity was exhibited just below the nodule clusters and H^+ was released in association with active nodules (Terry *et al.*, 1991; Terry and Jolley, 1994).

In split-root experiments (Terry *et al.*, 1991), soybeans were nodulated by *B. japonicum* only on one half of the root system and H^+ and reductant release appeared only on the nodulated side of T203 split-root systems. In another split-root experiment, T203 soybean was inoculated with combinations of effective or ineffective strains of *B. japonicum* (Terry and Jolley, 1994). The root nodules that formed with the ineffective *B. japonicum* strain were similar in number and shape to those on roots inoculated with the effective strain, but hydrogen ion and reductant were not released. Similarly, plants inoculated with effective or ineffective strains, but provided with nitrate in the growth media to inhibit nitrogenase activity, lacked Fe-stress response and became severely Fe chlorotic.

These experiments clearly illustrate that Fe reduction and release of H^+ and reductants around nodules and roots of inoculated Fe-inefficient T203 soybean are keyed to nitrogenase activity (Terry and Jolley, 1994). A practical illustration of the interaction of N_2 fixation and Fe uptake is found in peanut cropping systems in northern China (Zuo *et al.*, 2004). In this region, monocropped peanut is highly susceptible to Fe deficiency, while peanut intercropped with maize is not. Using split-root methods, the effect has been shown to be due to improved Fe nutrition in both the peanut and in the root nodules themselves (Zuo *et al.*, 2004) and likely due to the ability to obtain Fe from phytosiderophores released by maize roots. Root nodules on the peanut plants were more active as a result of the improved Fe nutrition, independent of the effects of soil N supply.

5. MANAGEMENT OF IRON NUTRITION IN FIELD CROPS

Management of Fe nutrition is unique compared to most essential nutrients for which deficiencies are easily corrected by adding relatively inexpensive fertilizer. Although soil chemistry and environmental conditions result in various inefficiencies with these other nutrients, these deficiencies are generally corrected with adequate fertilizer addition (Tisdale *et al.*, 1993). Alternatively, Fe deficiencies are not easily corrected with fertilizers applied to the soil in typical fashion (Anderson, 1982; Chen and Barak, 1982; Cihacek, 1984; Clark, 1982; Lucena, 2003; Vose, 1982). This anomaly is due to the extreme nature of the equilibrium between the solid and solution phase forms of Fe in the soil. This relationship is so "one-sided" in alkaline soils, that essentially an infinitesimal concentration of soluble Fe resides in the soil solution from which roots draw nutrients (Lindsay, 1974; Lindsay and Schwab, 1982). Iron fertilizer materials dissolve and temporarily elevate solution Fe concentration, but this increased solubility is short-lived and the net gain in terms of Fe uptake for plants is essentially nil.

Iron deficiencies are often spatially variable and are related to soil characteristics and landscape position (Franzen and Richardson, 2000; Hansen *et al.*, 2003; Vempati and Loeppert, 1986). Areas of chlorosis tend to occur within portions of fields rather than across entire fields. Thus, managers must decide whether to address Fe-deficiency problems over all areas of a field or to explore management focusing on zones where Fe chlorosis is most likely to occur. Variable rate management of the practices listed in this section may prove to be economical solutions if expense of treatment for Fe chlorosis is limited to affected areas (Hopkins *et al.*, 2005; Franzen and Richardson, 2000). Management of Fe deficiency is also complicated by year to year variability associated with temperature and precipitation patterns. Thus, managers may be faced with decisions without even knowing if deficiencies will develop.

As evidenced by the diverse and vigorous flora growing in alkaline soils, plants have evolved mechanisms to facilitate Fe uptake into their roots despite the virtual non-existence of Fe in soil solution. As stated previously, field crop production often requires management practices designed to enhance Fe availability to plants susceptible to Fe chlorosis. These management options include various cultural or fertilizer practices and/or selection of species, varieties, cultivars, or hybrids that have the capability of mobilizing Fe for root uptake (Jolley and Brown, 1994). These "Fe-efficient" or chlorosis tolerant plants have either evolved naturally or have been bred to possess some of the mechanisms which enable Fe solubilization

and absorption under less than ideal soil conditions. Screening approaches and techniques have evolved for different crop species.

5.1 Variety selection and screening for tolerance

In general there are two methods of screening cultivars for resistance to Fe deficiency chlorosis. The standard is field screening, including greenhouse and environmental chamber evaluations, where chlorosis is rated among the varieties compared to a control. The second method is to quantify one or more specific physiological responses to induced Fe deficiency stress

Field screening has been and continues to be the primary technique for isolating soybean genotypes with resistance to Fe deficiency. This is typically done by growing lines or individual plants on calcareous soils with a known history of inducing Fe deficiency (Diers and Fehr, 1989). Visual observation and rating of chlorosis severity is then used to quantify the degree of resistance among lines. The most common visual rating is based on a scale from 1 to 5, with 1 = no chlorosis, 2 = slight chlorosis, 3 = moderate chlorosis, 4 = intense chlorosis, and 5 = severe chlorosis with some necrosis. In recent years, use of hand-held chlorophyll meters has been adopted as an alternative to visual ratings (Peryea *et al.*, 1997; Frenkel *et al.*, 2004; Hansen *et al.*, 2003). Successful field screening requires observations of multiple environments (site years). It is a labor and cost intensive process. Another major limitation is the variability in expression of Fe chlorosis within fields and among years. Numerous attempts to overcome this heterogeneity have been made, including controlling levels of soil water, organic matter, soil compaction, clipping stems or leaves, and even applying herbicide (Chaney *et al.*, 1989; Fehr *et al.*, 1983; Froehlich and Fehr, 1981; Ocumpaugh *et al.*, 1992; Piper *et al.*, 1986). For some species, especially legumes, application of excess NO_3^- has been made deliberately to exacerbate Fe deficiency and to promote its uniformity (Lucena, 2000).

An attempt to speed up selection and to reduce soil and climatic variability has moved researchers into greenhouse and environmental chambers in either potted soils (Fairbanks *et al.*, 1987; Ocumpaugh *et al.*, 1992) or in nutrient solutions (Chaney *et al.*, 1992a). Fairbanks *et al.* (1987) reported that potted soils under controlled conditions could promote consistent expression of chlorosis symptoms in soybean which were highly correlated to those observed in the field. This approach has been used successfully for identification of tolerance in some species (J. Goos, North Dakota State University, Fargo, ND, personal communication).

Nutrient solution screening has been tried successfully in a few dicotyledonous species (Lin *et al.*, 2000). Maintenance of low Fe (Jolley and Brown, 1987) and addition of bicarbonate (Coulombe *et al.*, 1984; Dragonuk

et al., 1989; Fleming *et al.*, 1984) are commonly used to exacerbate chlorosis. Chaney *et al.* (1992a, 1992b) reported accurate distinction among soybean and chickpea (*Cicer arietinum*) cultivars of varying resistance using solutions containing bicarbonate and high concentrations of DTPA. This approach has been adopted by some crop breeding programs to screen for chlorosis (Charlson *et al.*, 2004).

Measuring varietal differences in physiological response to Fe deficiency has been suggested as a more rapid and simpler method than field screening. In situ measurements under conditions that will exacerbate the response to low Fe identify differences in tolerance to Fe deficiency chlorosis.

Jessen *et al.* (1988) noted that early expression of Fe deficiency response appeared to be correlated to Fe efficiency. This observation is important in the theory behind screening by Fe reduction measurement. An Fe-reduction based screening approach exploits the early Fe deficiency response of resistant plants by measuring Fe reduction early in the plant life cycle and correlating this value to chlorosis resistance. Jolley *et al.* (1992) found that chlorosis susceptibility relates closely to the magnitude and timing of Fe reduction by soybean roots. The quantity of Fe reduced over days four through eight (after initiation of Fe deficiency treatments) correlated negatively with field chlorosis scores with $r = -0.86$. Measurement over longer lengths of time did not improve correlation, demonstrating that accurate assessment could be made in a relatively short time period. The accuracy of the Fe reduction screening test has been further improved by buffering nutrient solutions with $CaCO_3$ and withholding Fe from both germination or growth solutions (Stevens *et al.*, 1993) making quantification of Fe reduction a functional method of Fe deficiency chlorosis resistance screening in plant breeding. Krouma *et al.* (2003) found differences in Fe reduction among five cultivars of common bean related to hydroponically induced Fe chlorosis, but no field chlorosis relationships were established in their study. Ellsworth *et al.* (1997) successfully related root Fe reduction of 24 dry bean cultivars to Nebraska field chlorosis scores.

Measuring H^+ ion release as a function of Fe deficiency is potential means of screening for Fe-efficient dicots. Wei *et al.* (1994, 1995) reported a closer relationship between H^+ ion release and Fe-deficiency chlorosis resistance than between Fe reduction and such resistance in subclover (*Trifolium subterraneum*). Resistant cultivars exhibited an enhanced H^+ ion pump under deficiency conditions (Wei *et al.*, 1995; Loeppert *et al.*, 1995). Ellsworth *et al.* (1998) evaluated eight lines of dry bean and 11 of soybean and found that H^+ extrusion was increased in response to low Fe. However the relationship between the H^+ response and field chlorosis scores was not as strong as between Fe reduction and field chlorosis scores. The summation of H^+ release and Fe reduction did not enhance the relationship compared to

Fe reduction alone. Krouma *et al.* (2003) reported that relative tolerance of five common bean cultivars was associated both with high capacity to acidify and to reduce Fe, but no field rankings of Fe-chlorosis tolerance were reported. These reports suggest that more study would be needed before measuring H^+ extrusion could be considered an important component in a screening program in most crops.

Grass species and genotypes within species vary in susceptibility to Fe-deficiency stress. Brown and Jolley (1989) confirmed a close relationship between release of phytosiderophore and susceptibility or tolerance to Fe-deficiency chlorosis in two oat cultivars. Römheld and Marschner (1990) and Onyezili and Ross (1993) showed that release of phytosiderophores and susceptibility or tolerance to Fe-deficiency chlorosis in various species were closely related. Thus, quantification of phytosiderophore release has been proposed as an effective means of variety selection for oat and wheat (Hansen and Jolley, 1995; Hansen *et al.*, 1996; Jolley and Hansen, 1995). Similar studies with corn phytosiderophore release and Fe-deficiency chlorosis development in Nebraska cultivars was not as effective as with oat and wheat (Bernards *et al.*, 2002).

There has been a tremendous development in understanding the genetic control and regulation of Fe uptake mechanisms in plants (Schaaf *et al.*, 2004; Bauer *et al.*, 2004; Römheld and Schaaf, 2004). There is potential for the use of molecular and biochemical approaches in breeding programs as alternatives to viva-culture methods used to identify resistance to Fe deficiency chlorosis. However adoption of such technology in large scale breeding has been limited (Lin *et al.*, 2000).

Regardless of the screening/breeding method used, the identification and planting of chlorosis resistant cultivars is the most important method of controlling Fe chlorosis development in field crops. A concern of planting these chlorosis tolerant plants over the whole field is lower yield potential in parts of the field where chlorosis is not a problem. Currently, some regions plant with separate seed in different parts of the field, i.e. a chlorosis tolerant cultivar in projected trouble spots and a high yielding cultivar in the rest of the field (J. Goos, North Dakota State University, Fargo, ND, personal communication). Blending high yielding seed with chlorosis tolerant seed has been used (W. Fehr, Iowa State University, Ames, IA, personal communication). While efforts to develop high yielding chlorosis tolerant plants continue, precision farming methods may enable growers to plant different seed sources within the same field to maximize yield i.e. lower yielding chlorosis tolerant cultivars are planted in management zones where chlorosis probability is likely and high yielding cultivars are planted in zones where Fe deficiency is not a concern. This may be another approach to reducing the indirect yield losses from planting chlorosis tolerant plants.

5.2 Fertilizer and cultural management practices

Although the use of Fe-efficient plants is generally the best approach to preventing Fe availability problems (Goos and Johnson, 2000; Hansen *et al.*, 2003; Jolley and Brown, 1994), several fertilizer and cultural management strategies are also available for use alone or in combination, to prevent/-correct Fe deficiency, including: 1) lowering soil pH, 2) applying foliar Fe fertilizer or acid sprays, 3) applying chelated/complexed Fe fertilizers to the soil, 4) applying Fe fertilizer materials in a concentrated band near roots, 5) companion cropping with Fe efficient species, and 6) altering management of irrigation and drainage, fertility and seeding practices (Hopkins *et al.*, 2005).

5.2.1 Lowering soil pH

Artificially lowering the soil pH would seem to be a viable alternative for correcting Fe deficiency (Lucena, 2003; Olson, 1950). For example, a pH drop from 7.5 to 6.5 would result in a ten fold increase in hydrogen ion activity and an associated 1000 fold increase in the solubility of soil Fe minerals. However, the amount of acidifying material required to lower the pH in a highly buffered calcareous soil is so great that this option is not cost effective, except in the case of very high value crops or in situations where cost is less of an issue. It may take as little as one half metric ton per hectare of elemental S to lower pH of a poorly buffered sandy soil from the alkaline range to a neutral pH, but a highly buffered (high clay and/or lime content) soil may require several tons to achieve the same result (Tisdale *et al.*, 1993). Furthermore, irrigation water high in lime may serve to raise the pH back into the alkaline range over time. Elemental S is the most common acidifying agent due to cost, availability, and handling advantages (Tisdale *et al.*, 1993). Many other solid and liquid compounds can be used to lower soil pH, including: sulfuric acid, phosphoric acid, aluminum sulfate, ammonium polysulfide, and any other non-toxic, acidic material readily available (Horneck *et al.*, 2005). In soils of low buffer and relatively low excess lime, addition of ammoniacal fertilizers could result in lowering of pH and more available Fe (Whitney *et al.*, 1991).

5.2.2 Foliar sprays

Liquid solutions containing low rates of chelated or non-chelated Fe are often used to temporarily correct Fe deficiencies (Anderson, 1982; Godsey *et al.*, 2003; Goos and Johnson, 2000; Mengel, 1995; Pestana *et al.*, 2001; Randall, 1981; Reed *et al.*, 1988; Zaiter *et al.*, 1992). If these materials are

applied in a fashion that results in adherence to leaf surfaces, the Fe is absorbed for use by the plant (Mengel, 1995). The main advantage of foliar applied Fe is that the fixation reactions of Fe in alkaline/calcareous soils are avoided (Mengel, 1995).

Foliar sprays should be applied with some type of adjuvant that will aid in even distribution and adsorption of the Fe on the leaf surface, where it can then be absorbed internally. Applying Fe with excessively high amounts of water will result in poor leaf adherence. Iron sprays that fall from the leaf surface to the soil will form insoluble precipitates that are not highly bioavailable (Olson, 1950). Supplying Fe into the irrigation water may be equally ineffective, although chelated Fe (EDDHA and analogous) proven to work in calcareous soil may be the exception if the Fe rates are relatively high and water-application rate is low. Although excessive water tends to reduce the effectiveness of a foliar application, enough water should be applied with a foliar Fe spray to insure adequate leaf coverage for uniform surface treatment to achieve maximum effect and to avoid spotting.

Although foliar Fe application readily supplies Fe into plant leaf tissue, this method of correcting Fe deficiency is temporary (Anderson, 1982; Godsey *et al.*, 2003, Römheld and Marschner, 1986) and may have an overall negative effect by inhibiting the plant's natural ability to overcome Fe deficiency by minimizing the activity of the Fe stress response (Römheld and Marschner, 1986). No residual benefit in terms of Fe availability in the soil is realized with foliar sprays. In fact, the effect is limited to the tissue to which Fe is applied due to the immobility of Fe once it becomes a part of the cellular structure (Vose, 1982). New leaves emerging after foliar application will be Fe deficient, unless the plant's ability to solubilize soil Fe by root activity is enhanced by application or the soil and/or environmental conditions change in favor of Fe availability (Anderson, 1982).

Foliar sprays generally correct visual Fe deficiency symptoms readily. In some cases, the foliar spray also results in a yield increase for field crops, but, in other cases, the effect is minimal. Randall (1981) states that a foliar spray of FeEDDHA effectively corrects Fe chlorosis in soybean, but the result may not always provide an increase in yield. Goos and Johnson (2000) found that foliar sprays of FeEDTA generally corrected Fe deficiency chlorosis in Fe-inefficient soybean varieties, resulting in yield increases at some, but not all locations tested. A yield increase was also observed in the Fe-efficient soybean variety at one location with a foliar spray. However, their conclusion was that selecting varieties not susceptible to Fe deficiency was a better management tool than use of foliar sprays.

Caution is needed when using foliar Fe sprays. Excessively high rates will result in tissue necrosis. Ferrous sulfate is a common source of non-chelated foliar Fe, but many other Fe-anion combinations may also be used.

Many other forms of complexed (citrates, fulvates, lignosulfates, gluconates, etc.) and chelated (EDDHA, EDHA, HEDTA, DTPA, etc.) Fe sprays are also available (Lucena, 2003). Foliar acid sprays (such as dilute sulfuric acid) have also been used to correct Fe deficiency by releasing physiologically inactive Fe already found in the leaf tissue (Sahu *et al.*, 1987). However, this method is not always as effective as foliar sprays containing Fe (Pestana *et al.*, 2001). Although only field-tested materials from reliable companies with adequate labeling instructions should be used for any fertilizer material, injuries from foliar sprays are relatively common and caution is particularly important with this management approach.

Single foliar Fe sprays can be cost effective, but there is often a need for repeated applications to make significant yield impacts on field crops (Anderson, 1982; Godsey *et al.*, 2003). Multiple applications may make this management approach impractical, especially for relatively low value field crops (Anderson, 1982). Mixing Fe fertilizer with pesticides or other fertilizer materials that are already planned to be foliarly applied makes the Fe application more cost effective (Mallarino *et al.*, 2001). However, caution must be exercised when tank mixing Fe with other materials, as the combination may result in inactivation of pesticides or precipitation of compounds that may result in plugging of the spray equipment.

5.2.3 Chelated and complexed Fe fertilizers

As mentioned previously, applying inorganic Fe fertilizer to soil is generally ineffective. Iron combines with anions in the soil to form compounds that are highly insoluble (such as ferric hydroxide) under alkaline pH conditions. Combining Fe with chelates or organic complexing compounds can reduce the rate of formation of these insoluble Fe compounds (Lindsay, 1974; Lucena, 2003; Vempati and Loeppert, 1988). An effective chelate essentially grasps Fe and keeps it in soil solution until it is either taken up by plants or microbes. Unfortunately, it may also be chemically or microbially decomposed, strongly bound by soil minerals or organic matter, or inactivated through the exchange of Fe with another cation (Álvarez-Fernández *et al.*, 2002; Lindsay, 1974; Lucena, 2003; Winkelmann *et al.*, 1999). The effectiveness of an Fe chelate compound may also be limited due to leaching, as synthetic chelates tend to be negatively charged and, as a result, are repelled by the negatively charged soil (Abadía *et al.*, 2004; Álvarez-Fernández *et al.*, 2002; Lucena, 2003; Winkelmann *et al.*, 1999). If excessive irrigation and rainfall does not leach the chelate from the root zone, the effectiveness of chelated Fe often lasts long enough to provide the plant with the Fe that it needs during the early part of the growing season when Fe deficiencies are generally most severe

(Álvarez-Fernández *et al.*, 2002; Lindsay, 1974; Reed *et al.*, 1988; Sahu *et al.*, 1988). In addition to the impact of leaching, decomposition, and inactivation, the effectiveness of synthetic chelates is influenced by factors such as amount and type of clay and salt in the soil (Siebner-Freibach *et al.*, 2004; Vempati and Loeppert, 1988).

Iron chlorosis is generally a problem in alkaline/calcareous soil, but most Fe chelates are not effective under these soil conditions. Only the EDDHA and analogous chelate forms (over 100 types are commercially available) have been shown to be effective for application to soils in which Fe deficiency is a problem (Abadía *et al.*, 2004; Goos and Germain, 2001; Lucena, 2003). Most other chelates (EDHA, HEDTA, DTPA, etc.) and complexes (citrates, fulvates, lignosulfates, gluconates, etc.) can maintain Fe in soil solution only temporarily and are not as effective as EDDHA and analogous chelates under calcareous conditions (Goos and Germain, 2001; Lucena, 2003). These other chelates may be used as foliar sprays or under mild deficiencies in non-calcareous conditions. The possibility of using Fe-chelates in slow-release forms has been evaluated for its ability to limit leaching losses and reduce Fe deficiency chlorosis (Goos *et al.*, 2004, Yehuda *et al.*, 2003). However, in a greenhouse evaluation, polymer coating for controlled release of Fe did not improve the effectiveness of many chelates and actually reduced the effectiveness of FeEDDHA (Goos *et al.*, 2004). Slow release technology likely has more potential for use with mineral Fe fertilizers, such as ferrous sulfate (Morikawa *et al.*, 2004; Singh *et al.*, 2004).

Although not as effective as EDDHA in calcareous soil, many organic compounds can complex Fe and potentially increase its concentration in soil solution in non-calcareous conditions or when applied at very high rates in calcareous soil (Lucena, 2003). These Fe complexing materials include both those specific Fe complexes, as mentioned previously, as well as non-specific humic substances found in biosolid amendments and in the fabric of the soil organic matter (Cesco *et al.*, 2000; Chen, 1996; Dahiya and Singh, 1979; Lucena, 2000; Mathers *et al.*, 1980; Olson, 1950; Pinton *et al.*, 1999; Pinton *et al.*, 2004; Nikolic *et al.*, 2004; Siebner-Freibach *et al.*, 2004; Thomas and Mathers, 1979). Similar to chelates, these organic materials contain carbon and, as such, are subject to chemical and microbial degradation over time. The rate of degradation of organic materials is highly dependent on soil temperature and is influenced by the activity and type of microbial species present and by soil moisture and oxidation state (Tisdale *et al.*, 1993).

Raw or composted biosolid materials (animal, human or industrial waste) generally contain large quantities of organically complexed Fe that can alleviate Fe deficiency if applied in large quantities (Anderson, 1982;

Mathers *et al.*, 1980). Inorganic industrial wastes may also serve as a source of Fe, although their plant availability is more akin to non-organic fertilizer materials (Wallace *et al.*, 1976). These organic biosolids are not as concentrated as most inorganic fertilizer but are applied in such large quantities to land as to serve as an excellent means of increasing complexed Fe in soil solution. However, unlike commercially available Fe chelates/-complexes, the availability of the Fe and other nutrients found in biosolids may be variable and dependent on microbial degradation of the material. In temperate climates, microbial activity is generally very low in the early part of the season when soil temperatures are cool and Fe deficiencies likely occur. Consequently, the availability of Fe from biosolid materials may be inadequate for early season use. It also is critical to manage biosolids properly to avoid N immobilization, toxicities to salt or specific ions/molecules, and/or problems associated with introduced pathogens and weeds.

The effect of organic matter in alleviating or preventing Fe deficiency is complex and not consistent. In addition to organic matter serving as a source of Fe, organic matter applied in large quantities can also result in "loosening" of the soil. Soil with lower bulk density has higher oxygen content and lower carbon dioxide concentration, which results in less negative impact of bicarbonate on Fe availability (Lucena, 2000). However, addition of easily degradable organic matter can temporarily have the opposite effect as the carbon dioxide level spikes as a function of microbial respiration during the mineralization process (Lucena, 2000). This results in the formation of bicarbonate in the soil, which reduces the bioavailability of iron.

Increased microbial activity is another potential benefit of carbon added *via* organic matter. Many microorganisms are known to release chelates known as siderophores which can mobilize Fe (Siebner-Freibach *et al.*, 2004). These siderophores are effective in solubilizing Fe for plant uptake and do not leach or decompose as readily as synthetic chelates (Siebner-Freibach *et al.*, 2004).

Although application of chelated or complexed Fe can effectively correct or prevent Fe deficiency, these materials are relatively costly to apply. Applying FeEDDHA chelates to soil is quite effective even at low rates. However, the cost of FeEDDHA is high and consequently is an option only for relatively high value crops. Similarly, application of biosolids and other sources of organically bound Fe require relatively high rates to be effective and transportation and application costs are prohibitive unless a local source is readily available and cost effective (Anderson, 1982).

5.2.4 Concentrated fertilizer

The cost of inorganic Fe fertilizer is relatively low, but, as discussed previously, broadcast application to the soil is generally ineffective. However, concentrating inorganic fertilizer in a band or point in the soil can effectively slow the rate of formation of insoluble Fe compounds within the concentrated fertilizer area, especially if it is acidic in nature (Godsey *et al.*, 2003; Goos and Johnson, 2000; Hergert *et al.*, 1996; Mathers, 1970). Studies in corn have shown that high rates of ferrous sulfate (~85 kg ha^{-1}) strategically placed in the seed row (Godsey *et al.*, 2003; Goos and Johnson, 2000; Hergert *et al.*, 1996) or in the path of roots effectively prevented Fe deficiency and resulted in significant yield increases (Goos and Johnson, 1999; Mathers, 1970; Mortvedt and Giordano, 1970). However, effectiveness is unpredictable (Godsey *et al.*, 2003; Mortvedt and Giordano, 1970) and likely depends on variable soil factors, including pH, lime content, salt concentration, organic matter, texture, temperature, moisture, and oxygen content (Bloom and Inskeep, 1986; Chen and Barak, 1982; Dahiya and Singh, 1979; Franzen and Richardson, 2000; Inskeep and Bloom, 1986, 1987; Moraghan and Mascagni, 1991; Morris *et al.*, 1990; Vempati and Loeppert, 1988).

The use of controlled-release Fe fertilizers applied as a band near or with the seed has been shown to ameliorate Fe chlorosis and improve yield of rice relative to similar placement of concentrated fertilizers without release control (Morikawa *et al.*, 2004; Singh *et al.*, 2004). This benefit may relate to a concentration of fine roots growing in direct contact with the controlled-release fertilizer granules, suggesting that fixation of released Fe by the soil is prevented by rapid root uptake. Controlled-release ferrous sulfate fertilizer did not improve growth of Fe deficient soybean, but a controlled release fertilizer with ferrous sulfate-ammonium sulfate-citric acid did (Goos *et al.*, 2004). Controlled release fertilizers may play an important role in managing Fe deficiency in some field crops, but the relatively high cost will limit its use.

It is entirely possible that a point injection below and to the side of each seed could also prevent Fe deficiency with a lower rate of fertilizer than reportedly used previously. Use of chelated/complexed Fe fertilizer sources would also be expected to work at much lower rates due to their relatively high efficiency. In theory these practices may be beneficial, but should be verified with research before adoption.

Impregnating chelated (FeEDDHA) Fe fertilizer directly on the seed has also shown some benefit in preventing Fe deficiency (Goos and Johnson, 2000; Karkosh *et al.*, 1988; Wiersma *et al.*, 2005) but the effect is not always realized (Goos and Johnson, 2000). Goos and Johnson (2000) found that

seed applied Fe effectively reduced Fe chlorosis and improved grain yield for soybean when applied in wide (76 cm) but not in narrow (15 cm) rows. However, their conclusion was that selecting varieties resistant to Fe deficiency was a better management tool than use of seed applied Fe.

Direct seed contact of any fertilizer salt requires low rates and caution, particularly with salt sensitive species and/or with soil or water already high in salts (Ayers and Westcot, 1985). Application of concentrated Fe fertilizer on or near the seed has been tested only on a limited number of crops, and rate/placement trials should be conducted to evaluate effectiveness and toxicity with other cropping scenarios.

5.3 Mixed cultivar and companion crop interactions

One management approach that has had some success in reducing the negative effects of Fe deficiency chlorosis is companion cropping of susceptible and tolerant species (Rombolà *et al.*, 2004; Zuo *et al.*, 2000, 2003, 2004). Explanations of how companion cropping may make Fe more available to susceptible species can be inferred from several studies done in controlled conditions, but their significance is less understood in soil culture (Bar-Ness *et al.*, 1991; Crowley, 2001; Hopkins *et al.*, 1992a, 1992b; Tagliavini *et al.*, 2000; Tagliavini and Rombolà, 2001). These studies illustrate that improvement in Fe uptake could result from a number of interacting factors such as improved root growth for more extensive root/soil interface and concomitant increased reductase activity, H^+ release, phyto-siderophore release, and/or increase in microbial populations or change in composition to favor Fe solubility. For example, in a greenhouse experiment conducted on calcareous soil (Zuo, 2003), serious chlorosis development was observed in peanut in monoculture compared to mixed culture with maize. Peanut growth in mixed culture resulted in modified root morphology (more lateral roots, longer and thinner roots and better defined rhyzodermal transfer cells in the subapical root zone—the site of Fe reduction/H ion release) and altered microbial populations (fewer bacteria and thicker mucigel layers on the root surface of mixed cropped peanut). Roots of peanuts in mixed culture exhibited consistently more Fe^{3+} reduction per g of root and over a longer period of time than peanuts in monoculture. These rhizosphere changes resulted in more total Fe in young and primary leaves, stems and roots and higher chlorophyll concentrations in all leaves and markedly higher HCl-extractable Fe in young leaves in mixed culture. Thus, both morphological and physiological characteristics of peanut roots seem to be associated with overcoming Fe deficiency chlorosis in this mixed-species system. Further, it was well documented that under mixed culture there was greater uptake of Fe by root nodules of peanut and that this resulted in an

increase in N_2 fixation independent of the effects of N fertility status (Zuo, 2004). Improved growth of peanut has been observed under field conditions, even when only one row of maize was planted between seven rows of peanut (Zhang, 2004).

5.4 Management of irrigation and drainage, fertility and seeding practices

Cultural practices often impact the severity of Fe chlorosis and manipulation of these practices can be effective remedies. Interacting management issues include poor drainage, compaction, salinity, fertility, irrigation, seed, and herbicides.

Iron chlorosis is frequently observed in poorly drained portions of a field (Bloom and Inskeep, 1986; Franzen and Richardson, 2000; Hansen *et al.*, 2003; Inskeep and Bloom, 1986, 1987; Moraghan and Mascagni, 1991). The chemical and biological reactions that occur under saturated conditions are complex and, as such, are difficult to predict (Marschner, 1986a). This effect is a result of poor root activity due to oxygen deficiency, cool soil temperatures, and increased bicarbonate due to carbon dioxide increase common in saturated soils (Bloom and Inskeep, 1986; Chen and Barak, 1982; Coulombe *et al.*, 1984; El-Shatnawi and Makhadmeh, 2001; Inskeep and Bloom, 1986, 1987; McBride, 1994; Moraghan and Mascagni, 1991). Root tissue and Fe solubilizing microbes will likely have reduced ability to implement Fe stress response mechanisms under oxygen and temperature stress (Marschner, 1986a, 1986b). Furthermore, chemical reactions and mineralization of Fe containing organic materials is reduced with cool soil temperatures common in poorly drained soils (Inskeep and Bloom, 1986; Marschner, 1986b). Compaction is often the cause of poor drainage, and plants grown on compacted soils often show Fe deficiency stress for this and other reasons. Increasing drainage in low lying areas can minimize compaction and improve Fe availability due to both improved oxygen/moisture ratio, as well as general root growth improvements. Tillage of currently compacted areas can also alleviate compaction and result in improvement with regard to Fe availability, although the effects are often short-lived in many soils (Tisdale *et al.*, 1993).

The opposite effect is observed less frequently with the severity of Fe chlorosis less in the poorly drained soil compared to normal areas. Possible explanations include reduced water stress in drought conditions, soil salt dilution, and/or increase in Fe reduction under anaerobic conditions (Bloom and Inskeep, 1986; Chen and Barak, 1982; Dahiya and Singh, 1979; Franzen and Richardson, 2000; Hansen *et al.*, 2003; Marschner, 1986a; Moraghan and Mascagni, 1991; Morris *et al.*, 1990). The bioavailability of Fe increases

in soils that are saturated for an extended periods (Chen and Barak, 1982; Lindsay and Schwab, 1982; Vose, 1982). As oxygen is depleted in a saturated soil, Fe^{3+} is reduced to the more plant available Fe^{2+} form. As the soil dries and oxygen concentration increases, the Fe is oxidized to the insoluble ferric form. Of course, field crops, other than rice, have reduced root growth and will eventually die under oxygen deprivation in the root zone (El-Shatnawi and Makhadmeh, 2001). Therefore, Fe chlorosis can be reduced for plants growing in poorly drained soils, but the effect may be mute or opposite if the plant root growth and biochemistry are inhibited by oxygen deficiency.

Although conflicting effects regarding Fe nutrition are observed under well and poorly drained soil conditions, it is generally advisable to implement cultural practices that result in good water drainage. Installation of drainage systems, minimizing or avoiding tillage and field traffic when soil is wet, reducing axle weight to minimize compaction, restricting traffic to the same rows, and/or addition of crop residues or other carbon rich materials are all good management practices that, among other benefits, may prevent water-logging of soils. Similarly, a properly designed irrigation system, with uniform water application applied in a timely fashion, can also prevent water-logging of irrigated soils and is also an overall good management practice.

In addition to drainage, modifying or managing irrigation water can impact development of Fe deficiency. High carbonate/bicarbonate concentration in soil water exacerbates Fe chlorosis (Chen and Barak, 1982; Coulombe *et al.*, 1984; Hansen *et al.*, 2003; Inskeep and Bloom, 1986, 1987; Lindsay and Schwab, 1982; Moraghan and Mascagni, 1991; Morris *et al.*, 1990; Vose, 1982). Drainage should reduce soil bicarbonate formation in rainfed areas. Under irrigation conditions, acidifying irrigation water reduces bicarbonate concentration and slowly dissolves soil carbonates, which may result in improved Fe nutrition. The cost effectiveness of such management is questionable for low value field crops. Another water management issue is salinity. Accumulation of salts is known to be related to Fe chlorosis. Thus, management of soil salts through proper drainage and irrigation practices may serve to lessen the development of Fe deficiency (Dahiya and Singh, 1979; Hansen *et al.*, 2003; Morris *et al.*, 1990; Siebner-Freibach *et al.*, 2004).

Interactions with other nutrients also may improve or worsen Fe chlorosis. The form of N present in the soil may impact Fe nutrition (Farrahi and Aschtiani, 1972; Lucena, 2000, 2003). The nitrate forms exacerbate Fe deficiency. However, the majority of ammonium from fertilizer or other sources converts to nitrate within days in aerated soil, making management of Fe chlorosis by using an ammonium form of N fertilizer largely

impractical when all or most of the fertilizer is applied at or before planting. Otherwise, frequent in-season applications of ammonium based fertilizers may help to alleviate Fe chlorosis. Thus, although the presence and uptake of the ammonium ion tends to reduce Fe deficiency, its effect is rarely enough to be considered a stand alone management tool to resolve the problem (Lucena, 2003).

As discussed previously, excessively high rates of P, K, S, Zn, Mn, or Cu fertilizers can also induce Fe deficiency (Astolfi *et al.*, 2003, 2004; Brown and Tiffin, 1962; Chen and Barak, 1982; Cumbus *et al.*, 1977; DeKock *et al.*, 1979; Haleem *et al.*, 1992; Hughes *et al.*, 1990; Kadar and Elek, 1999; Lindsay, 1984; Marschner, 1986a; Miller *et al.*, 1990; Roomizadeh and Karimian, 1996; Singh *et al.*, 1996; Szlek *et al.*, 1990; Uvalle-Bueno and Romero, 1988).

A variety of crop management practices may also improve Fe nutrition. Increasing plant population through increased seeding rate or decreased row spacing is another practice associated with minimizing Fe deficiency (Goos and Johnson, 2000; Hansen *et al.*, 2003; Penas *et al.*, 1990), presumably due to a collective improvement of Fe-stress response mechanisms or enhanced root development. Delaying planting date in problematic fields for Fe chlorosis is another cultural practice that may be used to minimize Fe stress (Hansen *et al.*, 2003). Iron stress is more common in the early spring and is often alleviated as root/microbial activity increases, organic matter is decomposed, and soils warm and dry. Herbicide toxicity and root injury due to mechanical or pest damage are both known to accentuate Fe chlorosis (Franzen *et al.*, 2004; Hansen *et al.*, 2003; Marschner, 1986b). In general, any practice that results in optimized plant growth improves a plant's ability to withstand the effects of Fe limiting conditions.

All of the management tools listed in this section have been shown to correct Fe deficiencies, but none is infallible and many are impractical, unpredictable and/or too costly for various cropping scenarios. Planting Fe efficient varieties, cultivars, hybrids or species remains the single most important management tool for field crop production. However, the other strategies discussed herein may be required to alleviate Fe chlorosis in field crop production even when Fe tolerant species or cultivars are used, but should be tailored to specific cropping systems and environmental and soil conditions and, as always, verified through in-field research prior to widespread adoption.

6. SUMMARY

Iron deficiency is a yield limiting concern in many field cropping systems. Iron deficiency is most common in soybean, peanut, dry bean, sorghum, and rice and under unique situation with corn, wheat and oat. These crops vary in their ability to obtain Fe and even vary across cultivars within a species. Iron deficiency occurs most commonly in alkaline/ calcareous soils but is impacted by soil carbonates, salinity, soil Fe composition nutrient interactions, soil moisture content, and soil bulk density. The use of Fe-efficient (chlorosis-tolerant) plants is generally the best approach to preventing Fe availability problems. However, several fertilizer and cultural management strategies are also available for use alone or in combination to prevent or correct Fe deficiency, including: 1) lowering soil pH, 2) applying foliar Fe fertilizer or acid sprays, 3) applying chelated/complexed Fe fertilizers to the soil, 4) applying Fe fertilizer materials in a concentrated band near roots, 5) companion cropping with Fe efficient species, and 6) altering irrigation and drainage, fertility and seeding practices.

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Chapter 3

IRON NUTRITION OF FRUIT TREE CROPS

ADAMO D. ROMBOLÀ and MASSIMO TAGLIAVINI
Dipartimento di Colture Arboree, Alma Mater Studiorum, Università di Bologna, Viale Fanin 46, 40127 Bologna, Italy. E-mail: rombola@agrsci.unibo.it

Abstract: Although iron (Fe) needs by fruit trees are relatively low, Fe deficiency represents the main constraint for successful cultivation of fruit tree crops in calcareous and alkaline soils. Kiwifruit, peach and pear, several *Citrus* and *Vaccinium spp.* are very susceptible to Fe chlorosis, cherry and grape are relatively susceptible and apple is relatively tolerant. The typical Fe deficiency symptoms, i.e. the interveinal leaf yellowing starting from apical leaves which may progress and turn into necrosis, exhibit a temporal and spatial variability, requiring an efficient diagnosis system. Iron deficiency reduces yields and fruit quality and forces growers to adopt measures for controlling and preventing the development of Fe chlorosis. The most widely used Fe fertilizers are synthetic chelates, which do not represent a sustainable management approach due to high cost and potential pollution of the soil and water environments. The genetic approach to prevent chlorosis is based on the choice of tolerant rootstocks, which are known to activate mechanisms for improving Fe uptake under condition of low Fe availability. Unfortunately, for several fruit crops iron tolerant rootstocks have adverse agronomic characteristics (e.g. excessive vigor) which make their adoption unlikely in modern fruit industry. Alternatives to Fe chelates have been identified and need to be tested and adapted to different conditions: they should aim at the improvement of soil environment for root growth and activity and/or to the enhancement of Fe availability in the soil and in the tree.

Key words: intercropping, iron chlorosis, iron demand, rootstock, sustainability

1. IMPACT OF IRON NUTRITION ON FRUIT TREE PRODUCTION

Fruit tree iron (Fe) requirements are relatively low. However, several species, grown on alkaline and calcareous soils, are unefficient in absorbing

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and using Fe and become Fe deficient. Iron-deficiency induced leaf chlorosis is the main constraint for successful production of several fruit tree crops cultivated in many production areas worldwide. The incidence of Fe chlorosis is widespread in the Mediterranean part of the EU (Abadía *et al.*, 2004). In Spain it has been reported in the Ebro Valley (Sanz *et al.*, 1992), Andalusia (Pastor *et al.*, 2002) and the Valencia Community (Legaz *et al.*, 1995). In Northern Greece, Fe chlorosis occurrence has been reported in the Imathia area (Tagliavini *et al.*, 2000). In France, approximately a quarter of the vineyard soils are described as calcareous (Ollat *et al.*, 2003). In Italy, alkaline and calcareous soils are widespread in deciduous fruit tree orchards of the Po Valley (Tagliavini *et al.*, 2000) and in several areas cultivated with *Citrus* and grape in Southern Italy, like the plain of Catania (Fichera, 1968) and Puglia (Rusco and Quaglino, 2001). In the Anatolian Region of Turkey, the major pistachio area, around 70% of the orchards are located on soils with more than 30% total $CaCO_3$ (Tekin *et al.*, 1998). Some 21-42% of soils planted with *Citrus* in Florida (USA) are calcareous (Obreza *et al.*, 1993). Iron chlorosis symptoms have been reported for plum and pear on alkaline or lime soils of California (Reil *et al.*, 1978; Yoshikawa *et al.*, 1982), for *Citrus* in the Tucumán district of Argentina (Aso and Dantur, 1972), and for *Citrus*, grapes, guava and banana in the Maharashtra and Tamil Nadu States areas of India (Kannan, 1988).

Kiwifruit, peach and pear, several *Citrus* and *Vaccinium spp.* are the most susceptible fruit crops (Korcak, 1987). Cherry and grape are considered relatively susceptible and apple relatively tolerant to Fe deficiency. Species rarely affected by Fe-chlorosis, like olive (Korcak, 1987), may display chlorotic symptoms when cultivated under intensive management (e.g. irrigation) (Fernández-Escobar *et al.*, 1993). Within a single species, differences in Fe susceptibility among varieties have been reported: for example, the pear cultivars "Abbé Fétel" and "Bartlett" are susceptible whereas "Conference" and "Hardy" are more tolerant (Korcak, 1987; Tagliavini and Rombolà, 2001); in olive, "Leccino" and "Picual" are sensitive, while "Hojiblanco", "Manzanillo" and "Nevadillo-Negro" are considered tolerant (Alcántara *et al.*, 2003; Benítez *et al.*, 2002). The tolerance to Fe chlorosis of the planting material is mainly due to the performance of the rootstock (the genotype providing the root system) and the appearance of the symptoms may be reduced by grafting on Fe-efficient genotypes (Socias i Company *et al.*, 1995), as discussed later.

Iron nutrition issues have an economical impact on fruit industry as (1) Fe deficiency reduces yields and fruit quality and therefore the grower income and (2) the measures for controlling and preventing Fe chlorosis are often expensive, representing a significant fraction of total production costs.

The costs for curing or preventing Fe chlorosis vary according to the degree of the problem and the type of crop, and depend on the strategy applied and its intensity. For Fe chelates, the application of 30-50 g tree^{-1} of (soil-applied) commercial product is quite common in mature peach orchards (Abadía *et al.*, 2004); Table 3-1 reports indicative ranges of amounts of Fe chelates as suggested by fertilizer producers and recommended by selected advisory and extension services. Considering, for example, average prices for Fe-EDDHA-based commercial products of approximately 8 € kg^{-1} and standard tree densities, the annual costs for controlling Fe chlorosis are often in the range of 240-400 € ha^{-1} for peach, 200-250 € ha^{-1} for kiwifruit and more than 400 € ha^{-1} for high density pear cultivation systems and for *Citrus*.

Table 3-1. Example of ranges of doses of major commercial Fe-chelates (g tree^{-1}) as indicated for curative applications in the product labels and suggested by selected advisory and extension services in Italy and Spain.

Crop	Indicated in the product label g (Fe-EDDHA based products) tree^{-1}	Suggested by advisory and extension services g (Fe-EDDHA based products) tree^{-1}
Citrus	100-250[1]	60-100[2]
Grape	20-40[1]	5-30[3]
Kiwifruit	30-90[1]	12-50[3]
Olive	30-40[1]	20-30[4]
Peach	80-150[1]	23-30[3]
Pear	80-110[1]	15-40[3]

[1]Based on major commercial Fe-EDDHA products listed by Álvarez-Fernández (2000) and Barbieri *et al.* (1992); [2]from Legaz *et al.* (1995); [3]based on bulletins of the advisory service of Consorzio Agrario (Ravenna, Italy) and of the INTESA grower's association (Faenza, Ravenna, Italy); [4]Sanz M. (pers. comm.).

Studying Fe chlorosis in deciduous fruit trees is complicated by the biennial reproductive cycle that most species exhibit. Such physiological feature implies that the nutritional status of the tree during the vegetative season when flower buds are formed affects the number of buds and fruit set the following year. Adverse effects of Fe chlorosis on yields have been reported at least in *Citrus*, kiwifruit, olive, peach, pear and plum (Bañuls *et al.*, 2003; Elkins *et al.*, 2002; Pastor *et al.*, 2002; Sanz *et al.*, 1992; Tagliavini *et al.*, 2000; Yoshikawa, 1988; Yoskikawa *et al.*, 1982) and depend on the degree and the period in which leaf chlorosis develops, being in general more severe during blooming and fruit set. The influence of Fe deficiency on fruit quality is mainly indirect as related to suboptimal leaf metabolism and net photosynthesis (Chen *et al.*, 2004). However, fruits with a green colored pulp (like olives and kiwifruit) may be not suitable for trade market if chlorosis develops in the pulp (Minguez-Mosquera *et al.*, 1991; Scudellari *et al.*, 1998). Iron deficiency may also affect fruit metabolism as a result of a change in activity of Fe-containing enzymes (e.g. aconitase)

located in the fruit pulp. A separate chapter of the book deals with effects of Fe deficiency on fruit quality (Álvarez-Fernández *et al.*, 2006). It should be considered that Fe deficient trees are also more susceptible to pathogen attacks. It is possible that some changes in xylem composition occurring in Fe deficient trees, like the increase of organic acid concentrations (Lopez-Millán *et al.*, 2001; Rombolà *et al.*, 2002a) affect the biology of some pathogens, like *Erwinia amylovora*, which produce siderophores under Fe-deficiency (Expert, 1999; Raymundo and Ries, 1980).

2. SYMPTOMS AND CONDITIONS LEADING TO IRON DEFICIENCY

The typical Fe deficiency symptom is the interveinal leaf yellowing starting from apical leaves; at that stage, shoot growth rate had already been reduced. In few species like pear, Fe chlorosis is atypical and widespread on the whole leaf lamina including the veins (Abadía *et al.*, 1988). If Fe deficiency progresses and new leaves are formed, then chlorotic symptoms can be observed also in basal or median leaves. If trees are not treated, Fe deficiency turns chlorosis into necrosis and leaves are shed earlier. Measuring leaf chlorophyll in the laboratory or using portable photometers (SPAD and others) to estimate it are the methods better suited to assess Fe deficiency (Abadía *et al.*, 2004), providing chlorosis is not due to other nutritional problems. To be reasonably sure that chlorosis is due to Fe deficiency, leaves should be sprayed with effective Fe fertilizers and re-greening should be observed.

Chlorotic symptoms exhibit temporal and spatial variability: within the same orchard they may vary from year to year as a result of tree and environmental conditions. Wallihan *et al.* (1976) observed that sweet orange grown in $CaCO_3$ enriched soil developed Fe chlorosis at soil temperature of 16°C but not at higher temperatures (19 and 22°C), and estimated that one °C increase of soil temperature in the range of 16-22°C caused a 5% enhancement of leaf Fe concentration. Symptoms of Fe chlorosis in orchards often occur in spring and are triggered by: 1) heavy rains, which tend to saturate soil pores and enhance HCO_3^- concentrations; 2) a marked increase of air temperature stimulating shoot growth and Fe requirements and 3) soil temperatures below optimal values for Fe uptake. Iron chlorosis problems may progress during the orchard lifespan, as roots tend to grow deeper and likely explore calcareous layers, where Fe is less available (Korcak, 1987; Tagliavini and Rombolà, 2001). The differences of texture, $CaCO_3$, organic matter, aeration, etc. often found in the orchard soil even at few meters of

distance account, at least in part, for the spatial variability of symptoms of Fe chlorosis within a single year.

The total soil Fe content in many soils planted with fruit trees would be theoretically sufficient to meet Fe needs. However, several soil-related characteristics reduce Fe availability and/or uptake, therefore leading to the development of Fe chlorosis (Chen and Barak, 1982). The prediction of potential risks of future development of Fe chlorosis in a tree plantation is of great importance and should guide the choice of the rootstock to be used. Mistakes at this stage would make unlikely the achievement of satisfactory yields without agronomic and chemical means for correcting the chlorosis. Due to the number of soil factors that impair Fe nutrition, it is not always easy to predict the possible chlorosis development of a perennial crop on the basis of a single soil parameter. Most Fe in the soil is present as inorganic Fe, predominantly goethite, hematite and ferrihydrite, all poorly available for plant uptake under aerobic conditions and high pH soils (Lindsay 1974), so determining total soil Fe content is of limited use. A better estimate of soil Fe availability for tree root uptake is obtained by extracting part of the Fe pools by DTPA or ammonium oxalate. Soil pH is often a suitable tool but it is not sufficient when used alone. Total soil calcium carbonate is not particularly useful for predicting the development of Fe chlorosis either, while determining the $CaCO_3$ in the fine, clay-sized fraction of soil gives more reliable information, as it represents the reactive fraction of $CaCO_3$, able to build and maintain high levels of HCO_3^- in the soil solution (Inskeep and Bloom, 1986). Some criticism has been raised (Patruno and Cavazza, 1989) about the way the clay-sized fraction of $CaCO_3$, known as active carbonate or active lime, is estimated (Drouineau, 1942). Active lime in fact reflects the amount of free Ca, and it may happen that soils well endowed with gypsum apparently have higher values of estimated active lime than measured total calcium carbonate. Pouget (1974) found that the tolerance to active lime increases with the amounts of available Fe in the soil and developed an index named “Chlorotic Power Index”, where the amount of active lime is normalized to the amount of Fe extracted by ammonium oxalate.

3. IRON NEEDS AND UPTAKE

There are only few reports indicating the Fe requirement of fruit trees. It is generally assumed that due to its poor phloem mobility, trees can recycle a limited amount of Fe from year to year, and that most Fe needed for vegetative and reproductive growth is derived from root uptake during each season.

Iron demand of mature vineyards is in the range of 650-1100 g Fe ha^{-1} per year (Gärtel, 1993): net removal is mainly accounted by the amounts recovered in yields and pruning wood. For several flesh fruit crops (Tagliavini *et al.*, 2000) Fe removal in fruits are in the range of 1-10 g Fe T^{-1} of fruit. In *Actinidia*, fruit Fe influx occurs throughout fruit development but accumulation rate is higher during cell division phase (Clark and Smith, 1988); mature kiwifruit vines producing 27 T fruit ha^{-1} take up slightly less that 2 kg of Fe ha^{-1} $year^{-1}$ and allocate it mainly into the wood and leaves (Smith *et al.*, 1988). Annual removal of Fe for peach trees accounted for 1-2 g Fe $tree^{-1}$ (corresponding to about 1.0 kg Fe ha^{-1}) in a survey carried out in the North Eastern part of Spain (Abadía *et al.*, 2004) where most absorbed Fe returned to the soil through leaf abscission. Examples of Fe partitioning are reported for apple and orange in Table 3-2. Orange data refer to the Fe amounts recovered in mature trees of cv. Navelina on Troyer citrange (Legaz *et al.*, 1992) while apple data (Tagliavini and Menarbini, unpublished) report the total amount of Fe recovered in the perennial organs of a 6^{th} year old "Mondial Gala" trees on "M9" rootstock, as well as the cumulative amounts of Fe in the abscised leaves, harvested fruits and pruning wood. For apple, considering the tree density of 2632 tree ha^{-1} and an original amount of Fe in trees at planting of 416 mg $tree^{-1}$, it was estimated an average uptake 1.4 kg Fe ha^{-1} $year^{-1}$. Data indicate that the net export of Fe is relatively low and that about 30% of the amounts taken up are returned to the soil by leaf abscission and pruning. When estimating Fe budgets it should be considered that, at least in calcareous soils, significant amounts of Fe are precipitated outside the root system or trapped in the root apoplast (Kosegarten and Koyro, 2001). This might explain the large amount of Fe recovered in the fine roots of orange trees in spite of their relatively small biomass.

With a few exceptions, perennial fruit plants are dicotyledonous (Strategy I species), which acquire most Fe after an enzymatic reduction from Fe^{3+} to Fe^{2+} (Fox and Guerinot 1998), a process which is greatly impaired at pH above 6.0 (Ao *et al.*, 1985; Susín *et al.*, 1996). The knowledge of Fe nutrition in perennial fruit plants has greatly improved in the last decade. Studies on Fe acquisition mechanisms have indicated that similarly to herbaceous dicotyledonous species some woody genotypes are able to improve Fe acquisition through adaptation mechanisms such as the acidification of the root apoplasm, the increase of root enzymatic Fe reduction and the excretion of reducing compounds (e.g. caffeic acid). In general, it should be noted that the induction of the Fe chelate reductase (FCR) and its activity are lower in woody perennials than in many annual plants (Moog and Brüggemann, 1994;). The low FCR activity recorded in some Fe-chlorosis sensitive species (e.g. *Annona glabra* and *Vaccinium spp.*) likely depends on them being native to wetland areas, which are

characterized by acidic soils with high organic matter and Fe^{2+} concentrations, conditions all of them favorable to Fe uptake (Ojeda *et al.*, 2003; Poonnachit and Darnell, 2004). The level of Fe concentration in the root medium affects the induction of root response mechanisms to improve Fe uptake and in general, absolute absence of Fe is less effective than extremely low Fe concentrations (Gogorcena *et al.*, 2000).

Table 3-2. Tree biomass and iron content in mature orange (cv. Navelina grafted on Troyer citrange) and apple (cv. Mondial Gala grafted on M9).

Tree organ	*Citrus sinensis*[1]		*Malus domestica*[2]	
	Dry weight (kg tree^{-1})	Fe content (mg tree^{-1})	Dry weight (kg tree^{-1})	Fe content[3] (mg tree^{-1})
Fruits[3]	-	-	10.8	131
Immature fruits	10.7	394	-	-
Shoots (including leaves)	12.2	1284	-	-
Abscised leaves and pruned wood[3]	-	-	6.9	1033
Old leaves	10.9	1318	-	-
Trunk and branches	45.1	2121	3.2	363
Coarse roots	20.6	1133	1.9	1206
Fine roots	2.9	2538	0.9	899
Total	102.4	8788	23.7	3632

[1] Legaz *et al.*, 1992; [2] Tagliavini and Menarbini, unpublished; [3]Cumulative amounts in the abscised leaves, pruning wood and harvested fruits since planting.

Proton release by roots buffers the pH in the root apoplast by neutralizing HCO_3^-, thus providing better conditions for Fe reduction (Mengel, 1994). Excretion of H^+ into the rhizosphere likely has no major influence on soil Fe dissolution in calcareous soils (Hauter and Mengel, 1988). Iron tolerant and sensitive rootstocks for fruit trees and grape differ as to their ability to acidify the rhizosphere. A decrease of rhizosphere pH as compared to bulk soil pH has been recorded for *Pyrus communis* (Tagliavini *et al.*, 1995a), *Malus xiaojinensis* (Han *et al.*, 1994 and 1998), *Actinidia deliciosa* (Vizzotto *et al.*, 1999) and *Vaccinium spp.* (Brown and Draper, 1980). The differential activity and induction of the enzyme phosphoenolpyruvate carboxylase (PEPC) between sensitive and tolerant genotypes has led to the hypothesis that this enzyme (which incorporates bicarbonate, the main causal factor of Fe chlorosis (Mengel, 1994), into phosphoenolpyruvate), can be listed among the adaptive strategies developed by dicotyledonous to cope with calcareous soils (De Nisi and Zocchi, 2000; Lopez-Millán *et al.*, 2000; Ollat *et al.*, 2003; Rombolà *et al.*, 2002a). The PEPC activity results in an accumulation of organic acids in Fe deficient plants (Abadía *et al.*, 2002b).

4. ADOPTION OF TOLERANT ROOTSTOCKS

In practice, the choice of planting material in orchards or vineyards is more influenced by the expected yields and market acceptance of a given cultivar rather than by its degree of susceptibility to Fe chlorosis. Therefore the genetic approach to prevent Fe chlorosis is represented only by the choice of the rootstock (Socias i Company *et al.*, 1995). The use of Fe-chlorosis tolerant rootstocks represents a reliable solution to prevent Fe chlorosis symptoms in fruit trees. However, tolerant rootstocks are often susceptible to other stresses (biotic or abiotic), induce an excessive growth of the scion (e.g. *Pyrus communis* seedlings for pear and "Fercal" for grape) or show adverse effects by reducing fruit yield and quality (Tagliavini and Rombolà, 2001). *Pyrus amygdaliformis*, for example, is highly tolerant to Fe chlorosis but it is not used as rootstock in commercial pear orchards, due to the astringency imparted to the fruits of the grafted variety (Procopiou and Wallace, 2000). The peach x almond hybrid "GF677" represents a successful example of a Fe-chlorosis tolerant genotype, and is largely employed as a rootstock for peach. In avocado, the West Indian rootstocks (e.g. "Maoz") are preferred to genotypes of Mexican and Guatemalan origin, due to their higher degree of tolerance to Fe chlorosis (Salazar-García, 1999).

In general, tolerant genotypes display an enhanced ability to reduce Fe at the root level (by the FCR enzyme) and releasing protons into the rhizosphere under low external Fe availability. Root enzymatic Fe reduction and proton extrusion have therefore been used as biochemical markers for screening tolerant genotypes (Gogorcena *et al.*, 2004). The Fe-chlorosis tolerance of *Citrus spp*. (Manthey *et al.*, 1994; Treeby and Uren, 1993) and peach (Romera *et al.*, 1991) rootstocks, but not of olive genotypes (Alcántara *et al.*, 2003) was associated with the increase in the ability of reducing external Fe^{3+} under Fe deficiency. Recent studies indicate the possibility of using further biochemical parameters as the activity of root phosphoenolpyruvate carboxylase (PEPC) and the accumulation of organic acids (particularly citric acid) in the roots (Abadía *et al.*, 2002b; Ollat *et al.*, 2003; Rombolà *et al.*, 2002a). Classical methods of plant breeding and new biotechnologies are being adopted to incorporate traits of resistance to lime induced Fe chlorosis of wild relatives naturally growing in calcareous soils into new, easy to propagate, rootstocks. Table 3-3 lists the degree of susceptibility to Fe chlorosis of major rootstocks for *Citrus* spp. Similar information for other fruit trees and grape is given in Coombe and Dry (1988), Rom and Carlson (1987), Socias i Company *et al.* (1995), Moreno *et al.* (1995), and Tagliavini and Rombolà (2001).

Table 3-3. Example of susceptibility to iron chlorosis of main *Citrus* rootstocks.

Rootstock species	Common name	Degree of susceptibility	Reference
Citrus aurantium	Sour orange	Moderately tolerant	Geraci, 1994; Treeby and Uren, 1993
C. sinensis	Sweet orange	Susceptible	Treeby and Uren, 1993
C. limettioides	Palestine sweet lime	Moderately tolerant	Geraci, 1994
C. limonia	Rangpur lime	Tolerant	Manthey *et al.*, 1994; Treeby and Uren, 1993
C. macrophylla	Alemow	Highly tolerant	Manthey *et al.*, 1994
C. jambhiri	Rough lemon	Highly tolerant	Manthey *et al.*, 1994; Treeby and Uren, 1993
C. volkameriana	Volkamer lemon	Tolerant	Geraci, 1994
C. reshni	Cleopatra mandarin	Slightly susceptible	Geraci, 1994; Treeby and Uren, 1993
Poncirus trifoliata	Trifoliate orange	Highly susceptible	Geraci, 1994; Korcak, 1987; Manthey *et al.*, 1994
P. trifoliata x C. sinensis	Troyer citrange	Slightly susceptible	Korcak, 1987; Manthey *et al.*, 1994; Treeby and Uren, 1993
	Carrizo citrange	Susceptible	Geraci, 1994; Korcak, 1987; Manthey *et al.*, 1994; Treeby and Uren, 1993
P. trifoliata x C. paradisi	Swingle citrumelo	Highly susceptible	Geraci, 1994; Manthey *et al.*, 1994

5. REMEDIES FOR IRON CHLOROTIC ORCHARDS

5.1 Synthetic iron chelates

The prevention and the cure of Fe chlorosis in fruit trees have been traditionally approached through the application of synthetic Fe chelates (Lucena, 2003). Several Fe chelators are available, those mainly used being EDTA (ethylenediaminetetraacetic acid) and DTPA (diethylenetriaminepenta-acetic acid) characterized by a low stability constant and suitable for foliar applications, and EDDHA [ethylenediaminedi(*o*-hydroxyphenylacetic) acid], EDDHMA [ethylene-diaminedi(*o*-hydroxy-*p*-methylphenylacetic) acid] and EDDHSA [ethylenediamine-di(2-hydroxy-5-sulfophenylacetic) acid], with higher stability constants (Lucena *et al.*, 1996) and suitable for soil supply. Soil application of Fe chelates aims at enhancing Fe availability for uptake at the root level and represents an efficient prevention method, provided no

major problems of root absorption, transport and leaf utilization arise. Soil applied chelates are ineffective when applied too early in the spring, when soil temperature is too low or when root uptake is impaired by waterlogging. Field studies on the effectiveness of various soil-applied synthetic Fe-chelates have recently been performed by Álvarez-Fernández *et al.* (2003a and 2004). Foliar applications of Fe chelates are used as alternative to soil Fe supply or to integrate the latter to provide more rapid leaf Fe availability during specific phenological stages. Due to the low Fe mobility in the phloem, repeated leaf sprays in chlorotic trees should be made to meet the Fe requirement during rapid shoot development (Rombolà *et al.*, 2000).

Iron chelates are usually effective but do not represent a sustainable approach to prevent or cure Fe deficiency (Tagliavini and Rombolà, 2001). Soil applied Fe chelates are water soluble and easily leached out of the root zone if excessive irrigation regimes are applied or during the autumn-winter (Rombolà *et al.*, 2002b). A likely underestimated problem related to synthetic chelates is the potential of some of them to bind heavy metals (Grcman *et al.*, 2001). Some iron chelates have a scarce degradability in the soil (Nörtemann, 1999) and may cause toxic effects on soil microorganisms and mycorrhizae (Grcman *et al.*, 2001). Moreover, it has been reported that plants may take up Fe-chelates directly and EDDHA traces could reach the fruits (Bienfait *et al.*, 2004).

The efficiency of Fe uptake from chelates can be enhanced by improving the technology related to their distribution and choosing the best application timings. Repeated additions of small amounts of Fe chelates through the irrigation water (by drip or micro sprinkler systems) likely maintain optimal Fe availability in the portion of soil where most roots are located and reduce the risk of Fe chelate leaching. Field experiments with clementine (Bañuls *et al.*, 2003) indicate that the frequency of Fe chelate supply by fertigation during the vegetative season does not consistently affect yields and fruit quality. In kiwifruit, late summer-early fall applications of Fe chelates were more effective than those carried out before bud burst in preventing the occurrence of early spring Fe chlorosis, possibly through mechanisms of storage and successive spring remobilization (Tagliavini and Rombolà, 2001). Attempts have been followed to reduce the risk of leaching of Fe chelates by fixing them to an organic matrix (Yehuda *et al.*, 2003).

5.2 Other mineral and organic iron sources

Ferrous sulfate, applied to the soil or to the tree (leaf treatments, trunk injection), has been the major therapy against Fe chlorosis from the first description of this nutritional disorder until the introduction of Fe synthetic chelates, and is still widely used by fruit growers in some developing

countries, due to its low cost. If supplied alone, Fe(II)sulfate is of little or no agronomic value in calcareous soils, where the Fe^{2+} is subject to rapid oxidation. For example, Fe sulfate was not effective for curing Fe chlorosis in *Actinidia deliciosa* in a soil with a high $CaCO_3$ content (32%), while a quite complete recovery was achieved by Fe-EDDHA (Loupassaki *et al.*, 1997). The effectiveness of soil applied Fe sulfate may be improved by the addition of organic substrates able to complex the Fe (e.g. animal manures, sewage sludge, compost, peat, etc.). Plant extracts or plant residues (e.g. from *Amaranthus retroflexus*) enriched with Fe salts may represent a promising way to improve soil Fe availability in both field crops (Matocha, 1984; Matocha and Pennington, 1982) and in fruit trees (Rombolà, unpublished).

Soil injection of a synthetic Fe(II)-phosphate ($Fe_3(PO_4)_2.8H_2O$), analogous to the mineral vivianite, achieved a long-term prevention of chlorosis in pear (Iglesias *et al.*, 2000), olive (Rosado *et al.*, 2002), kiwifruit (Rombolà *et al.*, 2003b) and peach (Rombolà *et al.*, 2003c). Synthetic vivianite is relatively inexpensive and can be directly prepared by growers simply dissolving ferrous sulfate heptahydrate and mono-ammonium (or di-ammonium) phosphate in water (Rosado *et al.*, 2002). Vivianite particles range between 2-10 μm in length (Iglesias *et al.*, 2000) and, unlike Fe-chelates, are hardly mobile through the soil profile and remain at the depth of application. According to Rosado *et al.* (2002) the long-term effectiveness of vivianite depends on the poorly crystalline status of Fe oxides (ferrihydrite and lepidocrocite) resulting from the oxidation and incongruent dissolution of vivianite. The formation of these oxides mainly depends on the continuous removal of phosphate from vivianite (Roldán *et al.*, 2002). The vivianite suspension, besides being an effective Fe fertilizer, also contains significant amounts of N, which should be considered in the orchard fertilization program.

Blood meal is an organic Fe source containing 20-30 g of ferrous Fe kg^{-1}, chelated by the heme group of the hemoglobin. Blood meal is a by-product of industrial slaughterhouses and examples of its effectiveness have been reported (Kalbasi and Shariatmadari, 1993). According to Mori (1999), the incorporation of Fe from hemoglobin into the root cells may follow a similar mechanism as the uptake of Fe in animal cells (endocytosis). Under field conditions, the application of blood meal (70 g $tree^{-1}$) alleviated Fe chlorosis symptoms of pear plants (Tagliavini *et al.*, 2000). It should be considered that blood meal is one of the main N fertilizers allowed in organic farming.

Injection of Fe salts (mainly Fe^{2+} sulfate and Fe ammonium citrate) in liquid form into xylem vessels has been reported to alleviate Fe chlorosis in apple, pear, peach, kiwifruit and olive (Wallace and Wallace, 1986; Wallace, 1991; Fernández Escobar *et al.*, 1993). In spite of the prompt re-greening

and long-lasting effect (2-3 years), this technique is seen as an emergency procedure for curing severely chlorotic trees (Wallace, 1991) and may be only feasible for low density planting systems. Main difficulties are related to the risk of causing phytotoxicity on leaves when Fe concentration and time injection are not properly chosen. Solid Fe implants formulations may not induce leaf burning and have been successfully applied to control Fe chlorosis of Spain (Larbi *et al.*, 2003).

The increase of soil organic matter content greatly reduces the risk of Fe chlorosis. Animal manure, particularly from cow, has been traditionally used to enhance soil organic matter content and fertility in orchards. The beneficial effect of organic matter on Fe chlorosis prevention depends on the direct Fe chelating ability of the humic and fulvic substances and on the stimulation exerted by organic components on soil microbial activities and root growth. In addition, organic matter improves soil aeration and may prevent the re-crystallization of ferrihydrite to more crystalline oxides in high pH soils (Schwertmann, 1966). Manure and compost are excellent substrates for bacteria (e.g. *Citrobacter diversus*) producing powerful Fe siderophores (Chen *et al.*, 2000), that have an important role on Fe acquisition by roots (Crowley *et al.*, 1991, 1992). Masalha *et al.* (2000) have shown that destroying soil microflora by sterilization impairs of Fe nutrition of Strategy I and II plants. Several siderophores have been isolated from manure (Chen *et al.*, 1998; Crowley, 2001; Yehuda *et al.*, 2000).

Water extractable humic substances (WEHS) may complex Fe from Fe-hydroxides and make it available for root uptake (Cesco *et al.*, 2000; Pinton *et al.*, 1999; Varanini and Pinton, 2005). The beneficial effect of WEHS of Fe nutrition also depends on their stimulation of proton ATPase and root growth (Pinton *et al.*, 1999). Very low concentrations of Fe-WEHS complexes in nutrient solution induced a rapid re-greening of Fe chlorotic grapevines (Baldi *et al.*, 2004). The efficiency of soil applied organic matter is often improved by incubation with Fe salts before application. For example, Fe deficiency of orange trees was prevented by applying a combination of peat and ferrous sulfate to a small portion of the root zone, whereas the application of peat alone was less effective (Horesh *et al.*, 1986).

Foliar application of Fe salts alone or mixed with organic acids has been tested in several studies. Canopy-applied ferrous sulfate represents a valuable, inexpensive alternative to Fe-DTPA or EDTA (Abadía *et al.*, 2002a; Álvarez-Fernández *et al.*, 2004a; Pestana *et al.*, 2002; Rombolà *et al.*, 2002c). Since the existence of inactivated Fe pools in chlorotic leaves has been proposed (Mengel, 1994), the possibility of inducing leaf re-greening under field conditions by spraying acidic solutions (e.g. citric, sulfuric, ascorbic acids) has been studied (Aly and Soliman, 1998; Tagliavini

et al., 1995b). Kosegarten *et al.* (2001) have reported that spraying citric and sulfuric acid resulted in a decrease of apoplastic pH followed by leaf re-greening. Under field conditions, however, this approach causes only a partial recovery from chlorosis (Tagliavini *et al.*, 2000). Effective re-greening of severely chlorotic kiwifruit leaves has been achieved by foliar application of Fe(III)-sulfate in combination with citric acid, malic acid and sorbitol (Rombolà *et al.*, 2002c).

5.3 Management of the rhizosphere and soil pH

Although modifying the pH of the entire soil volume explored by the root system is impractical (Wallace, 1991), lowering soil pH in small spots might be feasible by either removing part of the original calcareous soil and replace it with acidified peat (Sommer, 1993) or by the addition of acidifiers to bring about a complete loss of carbonates (Kalbasi *et al.*, 1986; Wallace, 1991). Removing part of the calcareous soil in the root zone and replacing it by a new substrate of acidified peat, supplemented with Fe, ammonium sulfate and nitrification inhibitors, has been proposed to prevent the Fe chlorosis in orchards and named "Cultan" System (Sommer, 1993).

It is well known that whereas uptake of nitrate causes an alkalinization of the rhizosphere, a nitrogen nutrition based on the uptake of ammonium brings about a decrease of pH at root/soil interface ammonium (Kosegarten *et al.*, 1999). Nutrient solution studies have shown that nitrate nutrition alone induces Fe chlorosis in peach, which on the contrary can be prevented by the co-presence of even low concentrations of ammonium (Kosegarten *et al.*, 2004). Similar to what happens at the root level, the presence of nitrate in the leaf apoplast also contributes to the alkalinization of this environment (Kosegarten *et al.*, 2001), enhancing the risk of Fe immobilization in the free space. This might be a concern for those plants, like kiwifruit (Ledgard and Smith, 1992) and grape, which use nitrate for transporting N from the roots to the leaves, and for plantings receiving high nitrate-N fertilization rates.

5.4 Intercropping with graminaceous species

The way orchards are structured frequently included areas between tree rows that might host annual or perennial grasses. Graminaceous species (Strategy II plants) are able to increase soil Fe solubility by secreting Fe-chelating substances (phytosiderophores, e.g. mugineic acid, MA) in the rhizosphere (Takagi, 1976; Ma and Nomoto, 1996). It has been recently hypothesized that intercropping an annual or perennial dicotyledonous with graminaceous plants may improve the Fe nutrition of the former (Kamal *et al.*, 2000; Tagliavini *et al.*, 2000; Zuo *et al.*, 2000). Although genotypical

differences within Strategy II plants are reported and examples of Fe inefficient graminaceous exist (e.g. rice, Ma and Nomoto, 1996), the MAs-based mechanism for acquiring Fe in calcareous soils, where the pH is buffered between 7.5 and 8.5 (Loeppert *et al.*, 1994) is generally more efficient as compared to the Fe reduction-based mechanism (Strategy I).

While MAs secretion by major cereals has been extensively studied (Ma and Nomoto, 1996), information on their production from graminaceous species either naturally present or artificially introduced in the orchard floor have been provided only recently (Ma *et al.*, 2003; Ueno *et al.*, 2002 and 2004). Pot trials have shown the potential effectiveness of this approach to improve Fe nutrition of guava (Kamal *et al.*, 2000) and kiwifruit (Rombolà *et al.*, 2003a). The soluble MAs–Fe(III) complexes originated by the grass cover could be reduced and/or taken up by roots of fruit trees (Rombolà *et al.*, 2004; Cesco *et al.*, unpublished). It is likely that the presence of grasses in the orchard affects Fe nutrition not only due to the secretion of MAs but also as a consequence of changes in soil organic matter and pH.

While the grass sward is traditionally restricted to the orchard alleys, the adoption of the intercropping concept could require the extension of grass sward under the tree row. Although this approach is very promising and interesting from an ecological point of view, from the existing knowledge it does appear that obtaining a beneficial effect on tree Fe nutrition from the synlocation of their root with those of a Strategy II plant is quite complex. Grasses should be managed so that they improve soil Fe availability for the tree, which depends not only by their MAs secretion but also by their subsequent Fe-MAs uptake. Under field conditions the choice of the intercropping system suitable for preventing Fe chlorosis should also take into account several factors like water availability, light, temperature and soil texture.

6. FUTURE PERSPECTIVES

Iron deficiency is the main constraint for successful cultivation of several fruit tree crops in calcareous and alkaline soils around the world. Iron deficiency reduces yields and fruit quality and forces growers to adopt measures for controlling and preventing the development of Fe chlorosis. To meet the requirements of sustainable fruit production, these strategies should 1) have a low impact on the environment, 2) be feasible and 3) have low costs. The genetic approach to prevent chlorosis development is mainly based on the choice of the rootstock and there are expectations about the release in the next future of new genotypes from breeding programs.

Table 3-4. Multi-criteria comparison of different strategies to control iron chlorosis in orchards.

Treatments	Effectiveness	Persistence	Annual Costs	Environmental impact	Comments	References
Soil application						
Fe-chelates	High	Low-medium	Very high	High	Possible legal restrictions in the future	Abadía *et al.*, 2004; Álvarez-Fernández *et al.*, 2003a and b; 2005 Bienfait *et al.*, 2004; Lucena, 2003; Rombolà *et al.*, 2002b
Fe-sulfate	Low	Low	Low	Medium	High soil moisture reduces rapid oxidation of Fe^{2+}	Loupassaki *et al.*, 1997
Vivianite	High	High	Medium	Medium	High P content may limit application	Iglesias *et al.*, 2000; Rombolà *et al.*, 2003b and c; Rosado *et al.*, 2002
Manure	Medium-high	High	Low-medium	Very low	Effectiveness may be improved by the addition of $FeSO_4$	Tagliavini *et al.*, 2000
Compost	Medium-high	High	Low-medium	Very low	Effectiveness may be improved by the addition of $FeSO_4$	Pérez-Sanz *et al.*, 2002; Tagliavini *et al.*, 2000
Cultan System	Medium-high	High	High	Low	Easier to be applied before tree planting - application technology after planting should be improved	Jaeger *et al.*, 2000; Sommer 1993
Blood meal	Medium	Low	Medium	Very low	Suitable for fertigation	Kalbasi and Shariatmadari, 1993; Tagliavini *et al.*, 2000
Foliar application						
Fe-chelates	Medium	Low	Medium	High	Prompt response - scarce effectiveness in severe chlorosis - possible future legal restrictions	Abadía *et al.*, 2004; Álvarez-Fernández *et al.*, 2004a; Larbi *et al.*, 2001; Pestana *et al.*, 2002; Rombolà *et al.*, 2002c
Fe-sulfate	Medium	Low	Low	Low	Prompt response -scarce effectiveness in severe chlorosis –phytotoxicity on fruits at high rates	Abadía *et al.*, 2002a; Álvarez- Fernández *et al.*, 2004a; Pestana *et al.*, 2002; Rombolà *et al.*, 2002c
Fe complexed with organic compounds	Low-medium	Low	Medium	Low	Prompt response – effectiveness depending on complexing agent	Abadía *et al.*, 2002a; Álvarez- Fernández *et al.*, 2004a; Pestana *et al.*, 2002; Rombolà *et al.*, 2000 and 2002c
Trunk injections	Medium-high	High	Medium	Low	Risks of premature leaf abscission – long persistence- risk of pathogen infection	Abadía *et al.*, 2004; Fernández-Escobar *et al.*, 1993; Larbi *et al.*, 2003; Wallace and Wallace, 1986; Wallace *et al.*, 1991
Intercropping with perennial graminaceous species	Medium-high	High	Low-medium	Very low	Effectiveness influenced by competition between grasses and trees – regreening the year after grass establishment	Kamal *et al.*, 2000; Rombolà *et al.*, 2003a and 2004; Tagliavini *et al.*, 2000

Chlorotic symptoms, typical yellowing of interveinal leaf areas, exhibit a temporal and spatial variability, requiring an efficient diagnosis system, which in the future may be based also on aerial photographs and remote sensing to guide the distribution of differential rates of Fe-fertilizer according to the degree of severity of the chlorosis within a single orchard. The application of iron chelates is not a sustainable way to prevent and cure iron chlorosis from either economical and ecological perspectives, although the adoption of effective distribution technology may partially reduce both concerns. Alternatives to Fe chelates have been identified and need to be tested and adapted to different conditions; they should aim at the improvement of soil rizosphere environment for root growth and activity and/or to the enhancement of Fe availability in the soil and in the tree.

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Chapter 4

IRON DEFICIENCY, FRUIT YIELD AND FRUIT QUALITY

ANA ÁLVAREZ-FERNÁNDEZ, JAVIER ABADÍA and ANUNCIACIÓN ABADÍA

Departamento de Nutrición Vegetal, Estación Experimental de Aula Dei, Consejo Superior de Investigaciones Científicas (CSIC), Apdo. 202, 50080 Zaragoza, Spain. E-mail: mabadia@eead.csic.es

Abstract: Iron deficiency is a major constraint for many fruit crops grown on calcareous soils. Iron deficiency is often assumed tacitly to affect negatively both fruit yield and fruit quality, but to our knowledge no review has been done so far on these specific issues. This review discusses first the negative effects of Fe deficiency in fruit yield, including as an example new data obtained for peach size, fruit number and yield per tree. Then, we discuss data available on the effects of Fe fertilization on the yields of different Fe-deficient fruit crops. Effects of Fe deficiency and Fe fertilization on flowering and fruit set are also reviewed, and the causes of the decrease of yield with Fe deficiency are discussed. The second part of the article discusses the published evidence on the effects of Fe deficiency on fruit quality in different crops. The effects of different Fe fertilization techniques on fruit quality are also reviewed. Fruits from Fe-deficient and Fe-sufficient trees may have similar size, firmness and color, but still show marked chemical characteristics affecting their organoleptic quality. Finally, future lines of research are also suggested.

Key words: iron deficiency; fruit; fruit quality; fruit tree; fruit yield

1. INTRODUCTION

Iron deficiency chlorosis is a worldwide problem in crop production on calcareous soils (Wallace and Lunt, 1960; Chen and Barak, 1982; Korcak, 1987; Marschner, 1995; Mengel *et al.*, 2001). Major annual crops affected by Fe deficiency include rice, soybean, field bean, pea, lupin, corn and sorghum. Crops producing fruits are affected very much by chlorosis,

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including tomato, raspberry, citrus, kiwifruit, pineapple, vines and many deciduous fruit tree species such as avocado, apricot, peach, plum, cherry and pear (Tagliavini and Rombolà, 2001; Rombolà and Tagliavini, 2005). Many fruit tree orchards, especially in Europe, are located on calcareous and alkaline soils, which favours the occurrence of iron chlorosis. Growers spend a large amount of money to control Fe chlorosis in fruit crops (Tagliavini *et al.*, 2000; Abadía *et al.*, 2004). For instance, in the Ebro river basin area, an important agricultural area in Northeastern Spain, approximately 45,000 ha of orchards are affected, and the cost of Fe fertilizers is more than 20 million US$ per year (Sanz *et al.*, 1992).

Iron chlorosis is a more complicated phenomenon in woody species than in annual crops, because long-distance Fe transport in these large plants is a more complex process (Tagliavini and Rombolà, 2001). Iron has to be taken from the soil and transported successively throughout root, trunk, branch and leaf tissues. This pathway involves a succession of still poorly understood biochemical processes, transport by small molecules that act as Fe chelators, and metal membrane transporters. When studying the effects of Fe deficiency on fruit characteristics, it should be taken into account that in fruit species the reproductive cycle starts with bud formation one year, followed by flowering, fruit set and fruit maturity on the following year (Toselli *et al.*, 2000).

In spite of the economic importance of Fe chlorosis in fruit tree crops, literature covering the effects of Fe deficiency on fruit yield and fruit quality has not been reviewed so far. The purpose of this chapter is to fill this knowledge gap.

2. EFFECTS OF IRON DEFICIENCY ON FRUIT YIELD

2.1 Iron deficiency decreases fruit yields

The general belief is that Fe deficiency decreases fruit yield in tree crops. This has been mentioned in recent reviews on Fe deficiency in tree crops (Tagliavini and Rombolà, 2001; Pestana *et al.*, 2003), although few specific references are usually mentioned in support of this view. Fruit yield losses can be due to decreases in the number of fruits per tree, decreases in fruit size or a combination of both factors.

Total yields of Fe-deficient and Fe-sufficient trees, growing side by side in the field, are indeed different. A typical example of the effect of Fe deficiency on fruit tree yield is shown in Figure 4-1. In a peach orchard, including a range of Fe-deficient and Fe-sufficient trees, each individual tree

was assessed during the year for leaf chlorophyll concentration (with the non-destructive SPAD technique), and at harvest time the number of fruits per tree, average fruit size and total yield per tree were recorded.

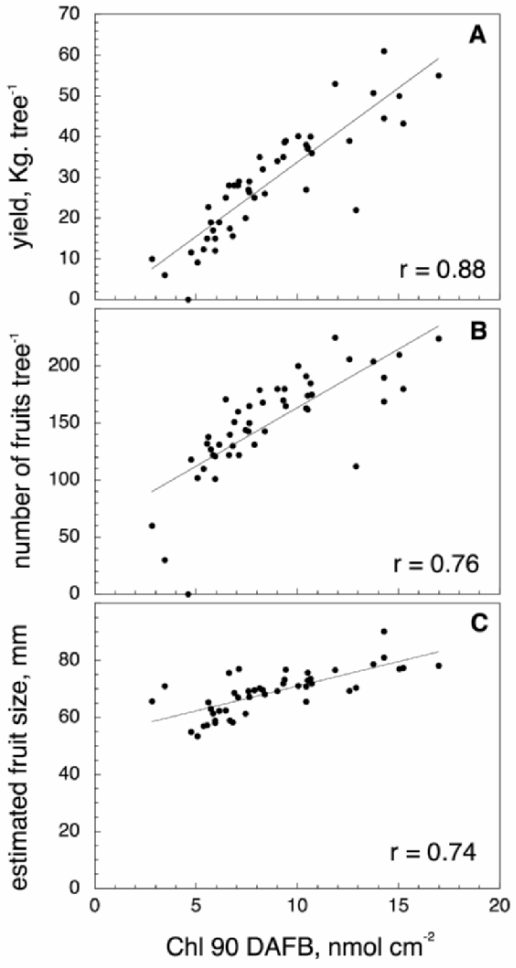

Figure 4-1. Relationships between leaf chlorophyll concentration (90 days after full bloom; Chl 90 DAFB) and total fruit yield per tree (A), number of fruits per tree (B) and fruit size (C).

Iron-deficient trees showed a marked decrease in yield (Figure 4-1A). One of the causes of this yield loss in Fe-deficient trees was a decrease in the number of fruits per tree as compared to the Fe-sufficient controls (Figure 4-1B). Down to chlorophyll values of 10 nmol cm^{-2}, the effect of leaf chlorosis in fruit number per tree was moderate; however, further decreases

in leaf chlorophyll from 10 to 5 nmol cm^{-2} led to 50% decreases in the number of fruits per tree (Figure 4-1B). Furthermore, in severely chlorotic trees the decrease in fruit number per tree could be higher than 80% as compared to Fe-sufficient, control trees (Figure 4-1B). Average fruit size was also decreased significantly by Fe deficiency, which further depressed fruit yield. Decreases in fruit size may be as large as 30% in severely deficient trees (Figure 4-1C). Consequently, fruit yield (in fruit fresh mass per tree) was considerably decreased by Fe deficiency, and even in moderately Fe chlorotic trees the loss in potential yield could be as high as 50% (Figure 4-1A).

This type of response, with Fe deficiency affecting (in decreasing order) total fruit yield per plant > number of fruits per plant > fruit size, could be quite common. In a recently published paper with two different peach cultivars growing in calcareous soils in Spain, Fe deficiency decreased leaf chlorophyll concentrations, number of fruits per tree and total fruit fresh weight per tree by 42-59%, 66-71% and 70-79%, respectively (Álvarez-Fernández *et al.*, 2003a). In this experiment crop yield decreased with chlorosis approximately from 50-60 to 10-12 t ha^{-1}. Significant decreases in average fruit weight, however, were found only in one cultivar (Álvarez-Fernández *et al.*, 2003a). A similar response in fruit yield with Fe deficiency was already found in the pioneering paper by Lyon *et al.* (1943), since deficiency resulted in no changes in tomato average fruit weight, but caused a 72% decrease in number of fruits per plant and a 77% decrease in total fresh weight yield per plant.

Decreases in yield with Fe deficiency chlorosis have also been found to occur in other fruit crops. In pear, Fe deficiency decreased leaf chlorophyll concentrations, number of fruits per tree and total fruit fresh weight per tree by 36, 72 and 64%, respectively (Álvarez-Fernández *et al.*, 2005). This indicates that in some conditions pear fruits can be larger in Fe-deficient trees than in Fe-sufficient ones (perhaps associated with a decrease in fruit set when Fe deficiency occurs at flowering). A severe reduction of total yield per tree was associated with a decrease in leaf chlorophyll concentration in peach, pear and kiwifruit trees growing in calcareous soils in Italy (Rombolà *et al.*, 1999). Fruit yield also correlates with other parameters used in the prognosis of iron chlorosis, such as flower Fe concentration. This has been shown to occur both in peach in Spain (Igartua *et al.*, 2000) and in *Citrus* species in Portugal (Pestana *et al.*, 2001a). In Pinot grape, Fe-chlorosis caused 85% and 61% decreases in dry weight of berries with a sensitive and a tolerant rootstock, respectively (Bavaresco *et al.*, 2003).

2.2 Iron-fertilization increases fruit yields

Further evidence supporting the effect of Fe deficiency on fruit yields comes from Fe fertilization trials. Since it is well known that Fe fertilization increases yields in many field crops, such as groundnut (Papastylianou, 1993), lentil (Erskine *et al.*, 1993), ginger (Wilson and Ovid, 1993) and dry beans (Zaiter *et al.*, 1992), it is expected that it would also affect yield in fruit crops.

Any kind of Fe fertilization in Fe-deficient fruit crops usually increases markedly fruit yields in the year following that of the Fe application. For instance, low-pressure trunk Fe-sulfate injections in olive trees increased both leaf chlorophyll and tree yield by at least 2-fold (Fernández-Escobar *et al.*, 1993). The increase in yield caused by Fe-fertilization is often associated with an increase in the number of harvested fruits per plant. The number of fruits per tree was found to increase in Fe-deficient peach after carrying out several different Fe-treatments, including soil chelate applications and trunk solid injections (Heras *et al.*, 1976). Also in peach, soil Fe-chelate treatments increased yield per tree by up to 35%, and caused an 11% increase in fruit diameter (Rogers 1975, 1978). In pear, pressure trunk injections of a 1% Fe-sulfate solution increased crop load in the next season by 25-29% with minor changes in fruit size, implying that an increase in fruit numbers per tree did occur (Raese and Parish, 1984). Solid Fe-sulfate implants in branches can increase yields in the next two years 2- to 6-fold, with increases in the number of fruits of 2- to 4-fold and 15-34% increases in fruit size (Larbi, 2003). In kiwifruit, soil Fe-EDDHA treatments reduced markedly leaf chlorosis and increased yield 2-fold, again mainly through an increase in the number of fruits per plant (Loupassaki *et al.*, 1997). In strawberry, foliar sprays of Fe-EDDHA increased yield per plant, mostly by increasing the number of berries (Zaiter *et al.*, 1993).

In some experiments it has been found that Fe-fertilization can increase peach fruit yield even in the same growth season. For instance, in a strongly chlorotic orchard in Spain Fe-sulfate injections in branches increased leaf SPAD index by 70-90% and almost doubled fruit size in the same year of fertilization; it is important to note that in this experiment the number of peach fruits was maintained equal in control and treated branches by carrying out fruit thinning (Sanz *et al.*, 1997). Soil Fe-EDDHA treatments produced rapid increases in leaf chlorophyll, and in the same season of the treatment both fruit size and tree yield increased by 4 and 30%, respectively, over the values found in untreated, Fe-deficient trees (Pérez-Sanz *et al.*, 1997). Peach yields can also increase in the same year when solid Fe-sulfate implants in branches are made (Larbi, 2003).

Several early papers already mentioned yield increases in *Citrus* species with Fe fertilization (Sites *et al.*, 1953; Carpena *et al.*, 1957; Stewart and Leonard, 1957). Soil and foliar Fe fertilization caused major (3-fold) increases in the tree yields of Fe-deficient *Citrus aurantifolia*, associated with 30% increases in fruit size and 2-fold increases in the number of fruits per tree (El-Kassas, 1984). In *Citrus sinensis,* soil Fe-EDDHA treatments produced a 26% increase in fruit yield, without affecting fruit size or leaf chlorophyll (Pérez-Sanz *et al.*, 1997). Alva and Obreza (1998) indicated that both Fe-EDDHA and Fe-humate increased tree yields in *Citrus sinensis* and *Citrus paradisi* by 6-55% and 4-9% (depending on the year), respectively, whereas fruit quality was not affected. Recently, it has been reported that Fe fertilization increased leaf chlorophyll (by 20-30%) in foliage-treated *Citrus deliciosa* x *Citrus nobilis* (Pestana *et al.*, 2000) and *Citrus sinensis* (Pestana *et al.*, 2000; 2001b; 2002), and also in soil chelate-treated *Citrus clementina* (Bañuls *et al.*, 2003). In *Citrus deliciosa* x *Citrus nobilis* fruit size was increased by 32% by the Fe treatments (Pestana *et al.*, 2000), whereas in *Citrus sinensis* yield increases ranged from 12 (Pestana *et al.*, 2001b) to 35% (Pestana *et al.*, 2002), depending on the year. In *Citrus clementina*, Fe treatments increased tree yield by 20%, mostly through an increase in the number of fruits, which were also slightly (but significantly) smaller (Bañuls *et al.*, 2003).

In some occasions, it has been reported that Fe fertilization may have little effect on fruit yield. This may occur when fruits are harvested only a few months after the Fe-treatment, and also in orchards where the intensity of chlorosis is mild or where chlorosis is already marked but it has been developed only recently. For instance, several soil Fe-chelate treatments made in late May were able to increase leaf chlorophyll concentrations in Fe-deficient peach, but did not have any effect on yield and fruit quality (Álvarez-Fernández *et al.*, 2003b). A soil Fe-EDDHA treatment carried out at the end of March was able to regreen the foliage of moderately chlorotic peach trees, increasing leaf SPAD index by 24%, but increased very little yields or fruit size in August (Pérez-Sanz *et al.*, 2002). In the same paper, it was reported that soil Fe-EDDHA treatment increased slightly (6%) leaf SPAD index in orange trees showing some signs of chlorosis, again causing no significant effects on yield or fruit size. Trunk injections of Fe sulfate in peach trees increased leaf chlorophyll by 26-53% and tree yield by a non-statistically significant 11% (Fernández-Escobar *et al.*, 1993). In this case, however, the weight of fruits removed by thinning was significantly higher in treated trees (Fernández-Escobar *et al.*, 1993). A likely explanation for the lack of effect of Fe treatments in orchards affected by mild chlorosis is that moderately deficient trees were able to cope with Fe deficiency by using

carbohydrate (and possibly Fe) reserves stored in previous seasons, therefore not suffering yet crop yield decreases.

2.3 Iron deficiency, flowering and fruit set

It has been established that Fe fertilization on a given year increases the number of fruits in the following season. This may be due to a variety of reasons, including effects in flowering, fruit set and others. In pear, pressure Fe-sulfate injections increased markedly percent bloom the following year (Raese and Parish, 1984). Solid branch Fe implants in peach also increase markedly flowering and yield the following year (Larbi, 2003). In kiwi, bud burst rate was increased by Fe fertilization (Loupassaki *et al.*, 1997). In apple, pressure injections of Fe(II)-sulfate increased chlorophyll 4-fold and increased fruit set the following year dramatically (Barney *et al.*, 1984). In a subsequent experiment, the same group found that injections of several Fe-compounds increased bloom (measured as clusters/tree) in apple for at least two years, although injections in July were too late to affect flower differentiation (Barney *et al.*, 1985). In olive, it has been reported that the percentage of flowers lacking ovaries is increased by Fe deficiency (Pastor *et al.*, 2001).

2.4 Why does iron deficiency decrease fruit yields?

The rationale for the decrease in fruit yield is the decrease in assimilatory power caused by Fe chlorosis. Low chlorophyll content in young leaves (chlorosis) is the most obvious visible symptom of Fe deficiency, and has been extensively documented in the literature (see Abadía, 1992). The main reason for this lack of chlorophyll is that major amounts of Fe are needed to build the thylakoid membrane of the chloroplast, where chlorophyll is located in form of chlorophyll-protein complexes. As a consequence, in Fe-deficient leaves the amount of chlorophyll-containing thylakoid membrane is very low, the electron transport capacity is limited and in turn the capacity to fix C is decreased drastically. Therefore, the growth of root, stem, branches and leaves is severely restricted by Fe deficiency (Lyon *et al.*, 1943; Bindra, 1980; Shi *et al.*, 1993; Loupassaki *et al.*, 1997; Morales *et al.*, 1998; Mengel *et al.*, 2001). The decrease in leaf area in Fe-deficient trees will further decrease their assimilatory capacity.

As we indicated above, the new development of iron chlorosis symptoms in fruit trees is unlikely to affect immediately fruit characteristics, because trees could rely on carbohydrate and Fe pools stored in previous seasons. Both buds and flowers need Fe and other nutrients for development, but little is known so far on where these required nutrients come from. In a

preliminary study made in peach, a soil Fe-EDDHA chelate application in late winter did not cause any effect on flower Fe concentration, supporting that Fe in flowers may come from previously stored Fe pools, either in the branches or in the trunk (Abadía *et al.*, 2000). However, a sustained chlorosis over a prolonged time is indeed deemed to induce a depletion of carbohydrate and Fe reserves, consequently affecting bud development, flowering, fruit set and fruit development.

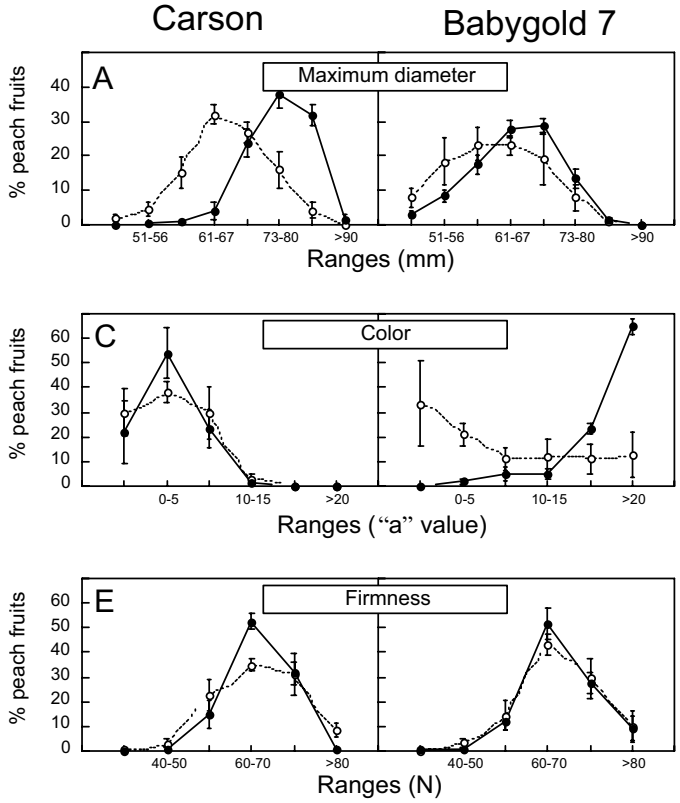

Figure 4-2. Distribution of the whole fruit population for fruit maximum diameter (A-B), "a" color coordinate (C-D) and firmness (E-F) in two varieties of peach. Open circles represent Fe-deficient trees and solid circles represent Fe-sufficient trees. Data are means ± SE (n = 4). Reprinted with permission from Álvarez-Fernández *et al.* (2003a). Copyright (2003) American Chemical Society.

3. EFFECTS OF IRON DEFICIENCY ON FRUIT QUALITY

3.1 Iron deficiency decreases fruit quality

Decreases in fruit quality due to Fe deficiency can be due to a number of factors, including decreases in fruit size, changes in fruit color, firmness and amount of juice, and also to changes in the fruit concentrations of chemical compounds such as organic acids, vitamins, phenolic compounds, etc., which may in turn affect organoleptic characteristics.

A first factor affecting fruit quality is fruit size, which often determines the value of the fruits and therefore the income of farmers. As discussed above, fruit size is markedly reduced by Fe deficiency, therefore decreasing the price of the product. For instance, in two peach varieties both maximum and minimum fruit diameter as well as fruit length decreased significantly with Fe deficiency (Álvarez-Fernández *et al.*, 2003a). Furthermore, the effect of Fe deficiency was more marked when the size distribution of the whole fruit population was used in the comparison, instead of using values for average size (Álvarez-Fernández *et al.*, 2003a). Ninety five percent of the fruits in Fe-sufficient 'Carson' trees had a maximum diameter larger than 67 mm, which is considered commercially appropriate, whereas in Fe-deficient trees only 47% of the fruits reached that size (Figure 4-2A; Álvarez-Fernández *et al.*, 2003a). In 'Babygold', where fruit size was smaller because fruit thinning was not done, commercially acceptable fruits were 43 and 28% in Fe-sufficient and Fe-deficient trees, respectively (Figure 4-2B; Álvarez-Fernández *et al.*, 2003a). In a similar study made with pear, however, most of the fruits were found to have a commercially acceptable size, both in Fe-deficient and Fe-sufficient trees (Álvarez-Fernández *et al.*, 2005).

Fruit color is another factor influencing fruit quality that could be affected by Fe deficiency. In a red-skin peach cultivar ('Babygold') Fe deficiency caused decreases in the mean "a" color coordinate and increases in the mean "L" and "b" color coordinates, although in a yellow-skin peach cultivar ('Carson') the means of the fruit color parameters were not affected significantly (Álvarez-Fernández *et al.*, 2003a). As it occurs in the case of fruit size, the distributions of the whole fruit population for color give more information than color mean values. In the red-skin cultivar, 33% of the fruits in Fe-deficient trees were green (with negative "a" values), whereas only 13% of the fruits had a strong red color ("a" values higher than 20); conversely, none of the fruits from Fe-sufficient trees were green, whereas 65% of fruits had a strong red color (Figure 4-2D; Álvarez-Fernández *et al.*, 2003a). In the yellow-skin peach cultivar, 54% of fruits in control trees had

"a" values between 0 and 5 (higher "a" values indicate higher maturity level), whereas in Fe-deficient trees only 38% of fruits were in that range (Figure 4-2C; Álvarez-Fernández *et al.*, 2003a). In a similar study made with pear, fruits from Fe-deficient trees also had more negative values for the "a" color coordinate than those from Fe-sufficient trees (Álvarez-Fernández *et al.*, 2005). This was possibly associated with a delay in fruit ripening at commercial harvest time. In green fruits, Fe deficiency can also lead to fruit yellowing (fruit chlorosis). This has been reported at least in pear (Raese *et al.*, 1986), olive (Mínguez-Mosquera *et al.*, 1991; del Campillo *et al.*, 2000) and kiwifruit (Scudellari *et al.*, 1998; Rombolà *et al.*, 2004).

Fruit firmness, a parameter of major importance for the quality of some crops such as pome fruits, could also be affected by Fe deficiency. Iron deficiency decreased the percentage of peach fruits with a firmness of 60-70 N in 'Carson' from the control value of 53% down to 35%, whereas in the cultivar 'Babygold' Fe deficiency did not change the shape of the fruit firmness distribution for the whole fruit population (Figure 4-2E-F; Álvarez-Fernández *et al.*, 2003a). In pear, Fe deficiency did not have any effect on the shape of the fruit firmness distribution for the whole fruit population (Álvarez-Fernández *et al.*, 2005).

Another important factor affecting fruit quality is fruit chemical composition. The first report indicating a significant effect of Fe chlorosis in chemical parameters related to fruit quality was the paper published by Lyon *et al.* (1943), showing that fruits from tomato plants grown with a limited supply of Fe contained 30% more ascorbic acid than fruits from Fe-sufficient plants. In *Citrus* spp., where citric acid concentrations in juice generally tend to decrease when the fruit matures, Fe deficiency causes relatively high citric acid concentrations in juice (Pestana *et al.*, 2000; 2001b; 2002).

Some of the effects of Fe chlorosis on fruit quality (but not all, see below) could be associated with a delay in fruit ripening. This was first suggested to occur in tomato (Lyon *et al.*, 1943), and it has been recently supported by data obtained for peach (Sanz *et al.*, 1997) and *Citrus* (Pestana *et al.*, 2001a). A delay in ripening will imply a less advanced stage in fruit development, a less intense color and chemical characteristics typical of under-ripened fruits.

3.2 Iron-fertilization increases fruit quality

As mentioned above, there is evidence in the literature that fruit size increases when Fe fertilization is carried out, therefore producing fruits with higher quality and better price. This has been shown to occur mainly in peach (Pérez-Sanz *et al.*, 1997; 2002; Sanz *et al.*, 1997).

Factors affecting fruit quality other than fruit size have been mainly studied in *Citrus spp*. The first trials with Fe-EDTA in Florida already indicated that Fe fertilization in Fe-deficient *Citrus* trees improved fruit quality, increasing soluble solids and volume of juice per fruit, slightly decreasing titratable acid in the juice and improving fruit color (Sites *et al.*, 1953). Increases in yields of Fe-deficient *Citrus aurantifolia* after soil and foliar Fe fertilization were associated with increases not only in fruit weight (30%) but also in juice contents (10%), and also to decreases in total acidity (10%), total soluble solids (25%) and ascorbic acid concentrations (10%), probably because of the dilution effect caused by a larger amount of juice per fruit (El-Kassas, 1984). Foliar Fe treatments repeated 10 times during the year (from August to February) increased the quality of March-harvested fruits in *Citrus sp.* through increases in fruit size and juice content and decreases in citric acid concentration, whereas total soluble solids increased significantly in tangerine (Pestana *et al.*, 2000), but not in orange (Pestana *et al.*, 2001b; 2002). These fruit quality improvements caused by Fe fertilization would mean a very significant 35-85% increase in gross income by the farmer (Pestana *et al.*, 2000; 2001b). Soil Fe-EDDHA treatments applied from April to September at different frequencies increased somewhat the fruit quality of November-harvested *Citrus clementina* (Bañuls *et al.*, 2003). Treatments tended to increase juice contents, total soluble solids, total acidity and color index, although differences were not statistically significant.

3.3 Are fruits of the same size and color different in iron-deficient and control plants?

A recent paper has provided evidence that the chemical composition of peach fruits having the same size, color and firmness, but coming from Fe-deficient and Fe-sufficient trees, is indeed different (Álvarez-Fernández *et al.*, 2003a). Both types of fruits had similar H^+ / titratable acidity ratios, suggesting that maturity was similar. The major finding in this study was a change in the total sugars to total organic acids (w/w) ratio, which decreased significantly with Fe deficiency, from 12 to 9 in the cultivar 'Carson' and from 10 to 7 in the cultivar 'Babygold' (Figure 4-3; Álvarez-Fernández *et al.*, 2003a). This change would likely modify the organoleptic characteristics of fruits, probably decreasing the value of the crop. In both cultivars, Fe deficiency generally caused moderate increases in organic anion concentrations, which were larger for succinate and quinate (35-52 and 33-48%, respectively), than for citrate and malate (17-20 and 11-12%, respectively; Álvarez-Fernández *et al.*, 2003a). Both the malate/citrate ratio and the sugar concentrations, including those of sucrose, glucose, fructose

and sorbitol, were unchanged by Fe deficiency in peach (Álvarez-Fernández *et al.*, 2003a). On the other hand, the concentrations of total phenolic compounds, soluble solids and acidity were not significantly affected by Fe deficiency, whereas vitamin C concentrations increased only in one of the cultivars ('Carson'). The influence of Fe deficiency on the mineral content of fruits was minimal (Álvarez-Fernández *et al.*, 2003a). It should still be tested in further experiments whether these chemical differences in apparently similar fruits can be due to some extent to a delay in fruit ripening.

A similar study with pear indicates that fruits having the same size, color and firmness, but coming from Fe-deficient and Fe-sufficient trees, also have different chemical composition (Álvarez-Fernández *et al.*, 2005). In this case, Fe deficiency caused only a small (non statistically significant) decrease in total sugars / total organic acids ratio, but increased markedly the malate/citrate ratio (Álvarez-Fernández *et al.*, 2005).

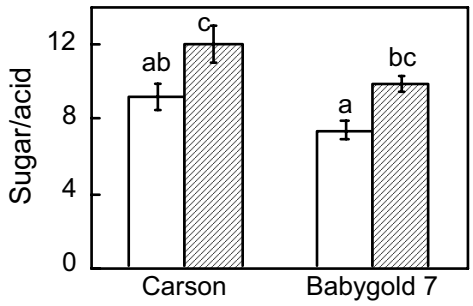

Figure 4-3. Effect of Fe deficiency on the total sugar to total organic acid ratio (w / w) in fruits of two peach cultivars. Open columns represent Fe-deficient trees and hatched columns represent Fe-sufficient trees. Data are means ± SE (n = 12). Reprinted with permission from Álvarez-Fernández *et al.* (2003a). Copyright (2003) American Chemical Society.

4. CONCLUSIONS AND FUTURE LINES OF RESEARCH

In summary, data available in the literature demonstrate that Fe deficiency chlorosis decreases markedly not only fruit yield but also fruit quality. Furthermore, any kind of Fe fertilization capable to control plant chlorosis does increase both fruit yield and fruit quality. The decrease in yield is often caused by a decrease in the number of edible fruits per tree, whereas fruit size is also affected, but to a smaller extent. Also, Fe-chlorosis increases the variability in fruit size, decreasing markedly the percentage of

commercially acceptable size fruits. It should be remarked that fruit size decreases are very important, because they often limit farmer crop cash returns.

In many of the published studies a fixed commercial harvest date has been used for both Fe-deficient and Fe-sufficient trees. Therefore, part of the decreases in fruit quality found at the commercial harvest date could be likely associated with the delay in maturity caused by Fe deficiency. Potentially, fruit harvest can be delayed by Fe chlorosis by perhaps two or more weeks, as it has been shown to occur in tomato, *Citrus spp.* and peach (Sanz *et al.*, 1997). Further experiments are needed to ascertain whether differences in quality could exist between fruits from Fe-sufficient trees taken at commercial harvest time and those of Fe-deficient trees taken some weeks later in the season. Of course, some of the deleterious effects caused by Fe deficiency, such as the decrease in fruit numbers per tree, would not be changed by harvest time. Other effects, such as those found in size, color and perhaps some chemical characteristics, could be reduced to some extent by delaying the harvest date in Fe-deficient trees, although fruits are likely to still be quite different in chlorotic and Fe-sufficient green trees.

Recent data indicate that fruits from Fe-deficient and Fe-sufficient trees having similar appearance (taking into account fruit size, color and firmness) had similar H^+/titratable acidity ratios (an index often used to assess maturity), but differed in chemical composition (Álvarez-Fernández *et al.*, 2003a). Peaches from chlorotic trees tended to have more organic acids than fruits from control trees, whereas sugar levels and mineral concentrations were little affected. As a consequence of these changes, Fe deficiency resulted in significant decreases in a commonly used peach fruit quality index, the total sugar/total organic acid ratio. The slightly higher phenolic compounds concentrations found in peaches from Fe-deficient trees may be associated with delays in maturity and could cause an increase in astringency, therefore decreasing tasting quality. Fruit phenolic compounds affect color and flavor, whereas vitamin C content contributes to fruit nutritive value. Therefore, changes in peach fruit chemical composition with chlorosis are likely to decrease to some extent their eating quality but also, surprisingly, to improve slightly their nutritional value. The malate/citrate ratio in fruit pulp did not change with Fe deficiency in peach, but in pear it was decreased markedly, and this could affect fruit organoleptic characteristics (Álvarez-Fernández *et al.*, 2003a; 2005). These changes in nutritional and tasting characteristics deserve further investigation.

Iron deficiency in trees develops progressively over the years unless some kind of Fe fertilization is used. This deficiency is assumed to deplete progressively the pools of Fe, carbohydrates and other nutrients in the tree. There are no studies, however, on the effects of the chlorosis evolution over

time on the yield and quality of the fruits produced. These studies would be critical to make an accurate estimation of the real impact of both chlorosis and fertilization practices in fruit yield and quality. Also, to gain a better insight into the effect of chlorosis in fruits it would be necessary to investigate in detail the pathways for the acquisition and transport of Fe during bud, flower and fruit development, which have been little explored so far (Toselli *et al.*, 2000). Studies should be also designed to investigate the specific effects of Fe chlorosis on bud development, flowering and fruit set.

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Chapter 5

SYNTHETIC IRON CHELATES TO CORRECT IRON DEFICIENCY IN PLANTS

JUAN J. LUCENA
Sección de Química Agrícola, Facultad de Ciencias C-VII, Universidad Autónoma, E-28049 Madrid, Spain. E-mail: juanjose.lucena@uam.es

Abstract: This article reviews the effectiveness of synthetic Fe-chelates to correct Fe chlorosis. Soil application of such compounds is usually a more efficient strategy to overcome Fe chlorosis of crops grown on calcareous soils as compared with treatments based on inorganic Fe salts or weak Fe-complexes. To understand the effectiveness of Fe-chelates, it is necessary to have knowledge on their stability, their chemical equilibria in soils and other factors that may affect their presence in soil solution. Sorption into soil surfaces should be also considered as a factor affecting Fe availability. Strategy I plants may obtain Fe even from the most stable chelates, but, among them, plants can take up Fe better from the less stable ones. Also, both the stereochemistry and the net charge of the Fe-chelates are important factors affecting their uptake by the plant. There is a wide variety in quality among commercial chelates, so that the use of reliable analytical methods is necessary to assess their purity and predict their behavior in field conditions.

Key words: chelates; chlorosis; iron; iron nutrition; stability

1. INTRODUCTION

Iron chlorosis in plants is an old worldwide problem occurring in areas of calcareous and/or alkaline soils (Marschner, 1995; Lucena, 2000; Mengel *et al.*, 2001; Tagliavini and Rombolà, 2001; Pestana *et al.*, 2003). In order to find a remedy to Fe chlorosis, the causes for this plant disorder should be reviewed. Iron chlorosis is due to several causes acting simultaneously. One is the low solubility of the Fe solid phase in soils (Lindsay, 1979). Although Fe is the most abundant nutrient in the mineral solid phase of soils (average of 3.8%), its presence in soil solution is extremely low (normally lower than

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1 $\mu g \cdot L^{-1}$). The concentrations of ferric ion (Fe^{3+}) and ferric species such as $Fe(OH)_n^{3-n}$ or FeX_n^{3-n} in solution are controlled by the thermodynamic solubility of the minerals present in the soil, which is minimal in calcareous soils (Lindsay, 1979). Reduction to Fe^{2+} and other ferrous soluble species are also low in well-aerated soils. Moreover, the dissolution rate of Fe from the solid phase, which is governed by the crystallinity (reticular energy) of the Fe solid phase, the surface of the solids and the presence of complexing agents in the soil solution (Schwertmann and Taylor, 1989), is normally very low. Iron uptake mechanisms and Fe distribution in plants may also be impaired under Fe chlorosis conditions. Bicarbonate affects all these processes due to its pH buffering capacity (Lucena, 2000), resulting in high pH in calcareous soils, where the solubility and dissolution rate of Fe are minimal. Bicarbonate also affects Fe uptake and distribution mechanisms in Strategy I plants.

2. REMEDIATION OF IRON CHLOROSIS

Remediation techniques for Fe chlorosis should affect at least one of the factors that induce it. The first possibility is to grow plants with improved Fe uptake mechanisms. This would be accomplished by traditional breeding (Cianzio, 1995) or in the future by obtaining genetically modified plants (Robinson *et al.*, 1999). However, this possibility should be regarded prior to planting, and once the crop is set other solutions must be considered.

Soil conditions can be improved to favour Fe dissolution and root development by adding organic matter (Chen, 1996). Organic materials can be applied with or without added Fe, in order to increase the concentration of Fe species in solution (Cesco *et al.*, 2000) and to increase the dissolution rate of the solid phases. There is also evidence of a direct effect of low molecular weight humic substances on Fe transport activities in roots (Pinton *et al.*, 1999). When large amounts of mature organic matter are added, soil structure can be improved, producing a better gas exchange in the soil, reducing the accumulation of CO_2 and thus of bicarbonate, and hence reducing the buffer capacity of the soil (Lucena, 2000). On the contrary, when fresh, easily degradable organic matter is added, an increase of CO_2 production and bicarbonate concentration can increase the induction of Fe chlorosis. The use of adequate organic matter may alleviate Fe chlorosis when factors inducing it are not very strong or crops are not very affected, but for the most susceptible crops this technique is not efficient enough.

Other soil conditioners are less effective. Locally increasing Fe solubility by acidification with mineral acids, ammonium salts, sulfur, and even with

some organic amendments, is not effective when the lime-bicarbonate buffer is present.

Iron can be supplied *via* foliar applications or trunk injection in order to increase its physiological availability inside the plant. Foliar sprays have been studied by several authors (Tagliavini *et al.*, 2000; Reed *et al.*, 1988), and in some cases good results were obtained. Álvarez-Fernández *et al.* (2004) studied the effectiveness of foliar fertilization with acids, $FeSO_4$ with and without acids and Fe-DTPA to re-green chlorotic pear trees, and they concluded that foliar fertilization cannot offer a good alternative for the full control of Fe chlorosis. They proposed that this could be a technique complementary to soil Fe-chelate applications. This is also a normal practice in crops where the use of chelates is too expensive. Trunk injection (Fernández-Escobar *et al.*, 1993) is expensive and mainly used in garden trees.

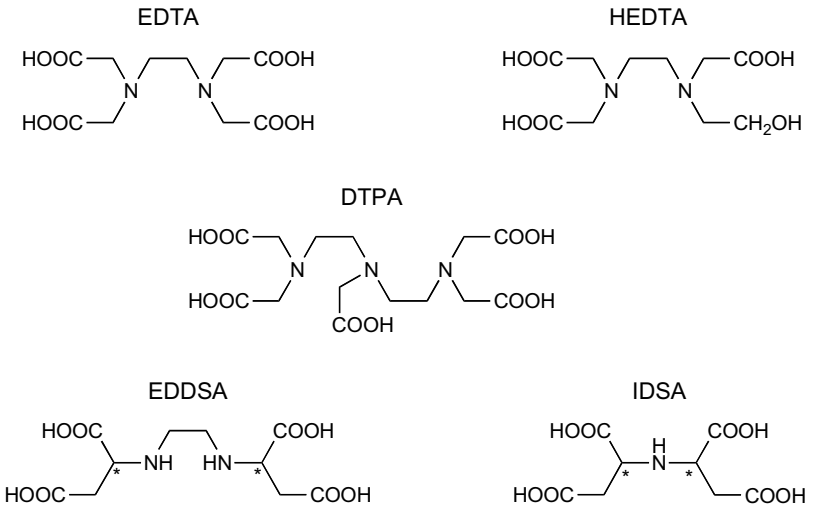

Figure 5-1. Chelating agents that form weak Fe-chelates. EDTA, ethylene diamine tetra acetic acid; HEDTA, 2-hydroxy ethylene diamine triacetic acid; DTPA, diethylene triamine pentaacetic acid; EDDSA, ethylene diamine disuccinic acid; IDSA, imino disuccinic acid. * Chiral carbons.

Fe fertilisers applied to the soil are widely used, and constitute the most common Fe chlorosis remediation technique. Inorganic Fe fertilizers are not efficient enough. Soluble salts (ferrous sulfate is the most common) precipitate rapidly to form more Fe hydroxide, similar to that already present in the soil in large amounts, whereas other ferrous crystalline products such as vivianite (Rosado *et al.*, 2002) or finely divided pyrite (Vlek and Lindsay,

1978) introduce into the soil a more soluble solid phase, with a large surface area, therefore increasing the concentration of Fe(II) and Fe(III) in solution.

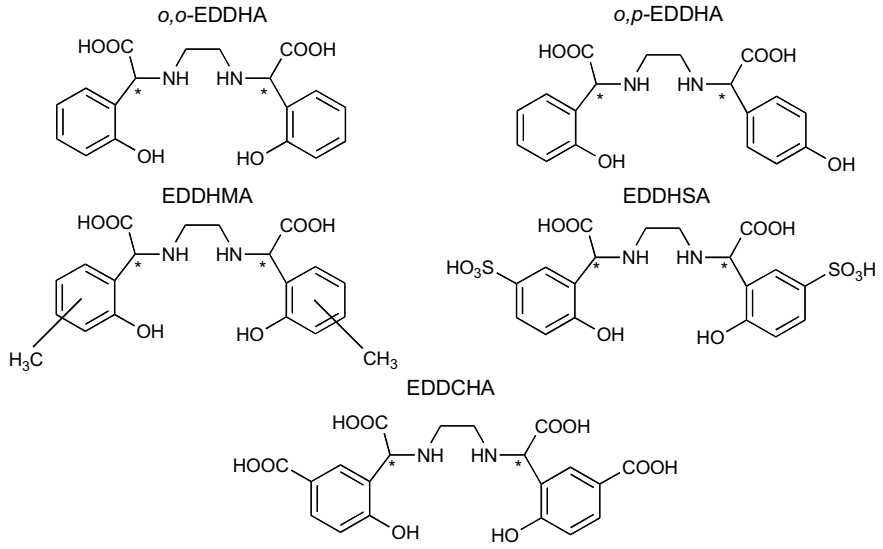

Figure 5-2. Poliamino-phenolate-carboxilic chelating agents present in commercial Fe fertilizers: *o,o*-EDDHA, ethylendiamine-di(ortho-hydroxy phenyl)acetic acid; *o,p*-EDDHA, ethylendiamine-N(ortho-hydroxy phenyl acetic)-N'(para-hydroxy phenyl acetic) acid, a compound present in commercial products along with *o,o*-EDDHA; EDDHMA, ethylendiamine-di(ortho-hydroxy methyl phenyl)acetic acid; EDDHSA, ethylendiamine-di(2-hydroxy 5 sulfonate phenyl) acetic acid (it should be noted that commercial products with EDDHSA also include condensation products); EDDCHA, ethylendiamine-di(5-carboxy 2-hydroxy phenyl) acetic acid. * Chiral carbons.

Complexes of Fe (with citrate, fulvates, lignosulfonates, gluconates, etc.) and low-stability synthetic Fe-chelates (with HEDTA, EDTA, DTPA and others, see Figure 5-1) can maintain Fe in the soil solution, with the exception of calcareous soils. These Fe compounds could be useful for foliar application or in nutrient solution, in conditions where chlorosis is not severe. Only the most stable chelates (*o*,*o*-EDDHA/Fe^{3+} and analogous, see Figure 5-2) are able to maintain Fe in the soil solution, and transport it to the plant root, in highly calcareous soils. Iron-chelates are the most efficient remedy to control Fe chlorosis, but they are expensive. In fact, only in Europe, more than 60 million US dollars per year are used to treat crops affected by Fe chlorosis with Fe-chelates (Álvarez-Fernández *et al.*, 2005). The most used and effective chelating agents are diamino-diphenolic-dicarboxylic acids, mainly *o*,*o*-EDDHA (ethylene diamine di-(ortho-hydroxy phenyl acetic) acid) and analogous (see Figure 5-2). Due to the high price, only cash crops

may be treated with these Fe-chelates (Chen and Barak, 1982), whereas areas and crops with low productivity are not supplied with these products. Improvements in the efficacy of Fe-chelates may reduce the application rates needed, and hence the cost of the treatments.

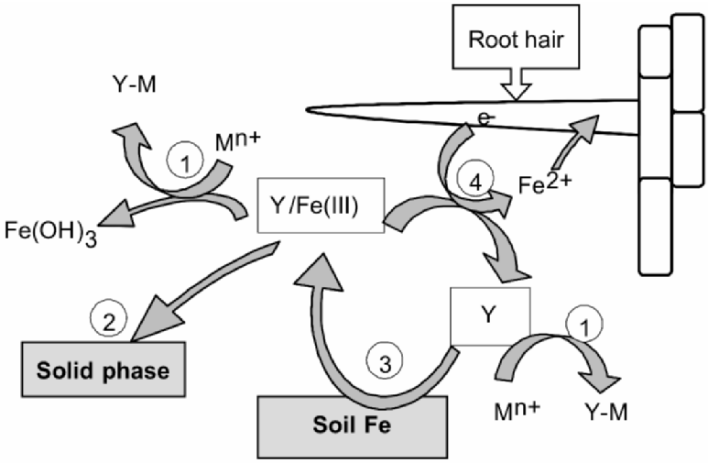

Figure 5-3. Schematic representation of the reactions of a chelate in the soil.

3. SYNTHETIC IRON CHELATES AS IRON CARRIERS TO THE PLANT

It has been hypothesized (Lindsay, 1995; Lucena, 2003) that Fe chelates serve as carriers for the soil native Fe, from the Fe containing soil minerals to the plant roots. This mechanism may be described in four steps (see 3 and 4 in Figure 5-3):

- Fe(III) must be released from the Fe-chelate, so that the plant can absorb it. As a consequence, the chelating agent is also released into the soil solution.
- The free chelating agent moves by gradient flow from the root surface to the soil solid phases.
- The chelating agent may dissolve native Fe present in the solid phases.
- The Fe-chelate must return to the root surface, by diffusion or mass flow.

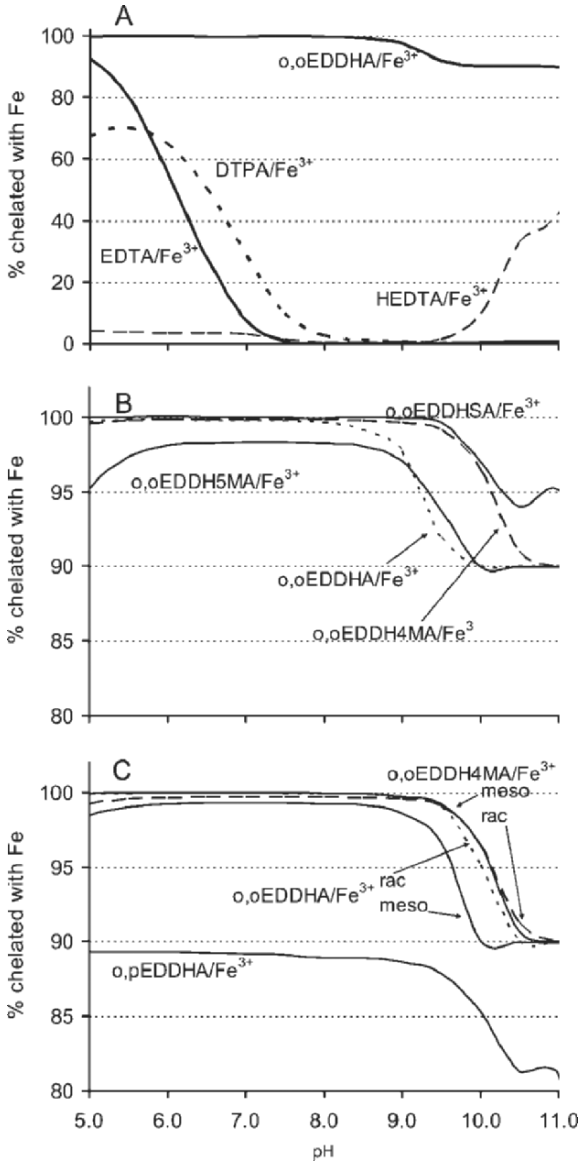

Figure 5-4. Stability diagrams for iron-chelates in soil conditions (after Lucena *et al*., 2005). A: Weaker chelates and *o,o*-EDDHA/Fe^{3+}; B: Strong chelates; C: Positional (ortho-para) and geometric (*racemic* and *meso*) isomers of *o,o*-EDDHA/Fe^{3+} and *o*,*o*-EDDH4MA/Fe^{3+}. Speciation conditions: 100 µM chelates, maximum soluble Cu^{2+}10 µM and solids defined as in Lindsay (1979).

Therefore, the efficacy of the chelate to carry Fe to the plant depends on the processes that the Fe-chelate or the chelating agent suffer in the soil plant system depicted in Figure 5-3: 1) the stability of the Fe-chelate and chelating agent in solution, 2) the adsorption of the free chelate onto the soil surfaces, 3) the rate at which the native Fe in the solid phase of the soil can be dissolved by the chelating agent, and 4) the ability of the plant to take up Fe from the Fe-chelate. These processes will be reviewed in the next sections.

4. STABILITY AND SPECIATION OF IRON CHELATES IN SOIL SOLUTION

Four processes may be involved in the reaction of Fe-chelates with soil that may reduce their presence in the soil solution:

- Fe-chelate or chelating agent degradation
- Replacement of the Fe by competing metals

  $FeY^{-} + M^{2+} + 3H_2O \leftrightarrows Fe(OH)_3 + MY^{2-} + 3H^{+}$
- Sorption of the Fe-chelate onto the soil surfaces
- Percolation (lixiviation)

Chemical degradation of Fe-chelates is slow in the dark (Hill-Cottingham, 1955; Lahav and Hochberg, 1975). Thus, they have been considered as non-environmentally degradable, despite the fact that no experimental data are yet available proving an accumulation associated with agronomic practices. New biodegradable chelating agents (see EDDSA and IDSA in Figure 5-1) have been recently introduced to substitute EDTA in other industrial uses (Nowack and VanBriesen, 2005), and it may be possible to use them in Fe chlorosis correction.

The other three processes are also important in soils. Chelates and chelating agents may react with other metals or protons. Only adequate chelating agents may complex Fe in soil conditions (Lindsay, 1979; Lucena *et al.*, 1988; Norvell, 1991; Yunta *et al.*, 2003a; Sierra *et al.*, 2004). Metal competition can be studied theoretically by means of chemical speciation programs, provided that thermodynamic data are available for all the possible reactions (Yunta *et al.*, 2003b; Lucena *et al.*, 2005). The early works of Lindsay (1979) are a good example. The theoretical stability of the most common commercial Fe chelates in soil conditions is shown in Figure 5-4. Only EDTA and analogous compounds are not stable enough to maintain chelated Fe in the soil solution. In the conditions found in soils at pH values below 9, which is a normal pH limit for agronomic practice, the stability of *o*,*o*-EDDHA/Fe^{3+}, EDDH4MA/Fe^{3+}, EDDH5MA/Fe^{3+} (the number in EDDHMA/Fe^{3+} describes the position of the methyl substitution in the aromatic ring) and EDDHSA/Fe^{3+} is sufficient to maintain all Fe in solution;

the stability sequence is, in decreasing order, EDDHSA/Fe^{3+} > EDDH4MA/Fe^{3+} > EDDH5MA/Fe^{3+} ≈ *o,o*-EDDHA/Fe^{3+} >> DTPA/Fe^{3+} > EDTA/Fe^{3+} > HEDTA/Fe^{3+}. The last three compounds cannot be used in calcareous soils, because the Fe-chelate is broken at pH values above 6, due to Zn, Mn or Ca substitution and the subsequent precipitation of Fe. For the most stable chelates, the stability sequence found in soils does not match the stability constant (log Kº) sequence (Yunta *et al.*, 2003b), which is *o,o*-EDDHA/Fe^{3+} (35.09) > EDDH4MA/Fe^{3+} (34.44) > EDDH5MA/Fe^{3+} (33.66) > EDDHSA/Fe^{3+} (32.79). This occurs because proton, Cu^{2+}, Ca^{2+} and Mg^{2+} competition do affect equilibria differently. Therefore, when comparing the stability of different Fe-chelates, all possible reactions affecting them should be considered in the chemical speciation, instead of comparing only the log Kº values.

o,*o*-EDDHA/Fe^{3+} and analogue molecules contain two diastereoisomers: *meso* and *racemic*. These diastereoisomers also behave differently. While *racemic*-*o*,*o*-EDDHA/Fe^{3+} presents a higher log Kº (35.86) than that of *meso*-*o*,*o*-EDDHA/Fe^{3+} (34.15), for EDDH4MA the opposite occurs (35.54 for *meso*-EDDH4MA/Fe^{3+} and 33.75 for rac-EDDH4MA/Fe^{3+}). Larger differences have been found among the hydroxyl-positional isomers of the chelates (Yunta *et al.*, 2003c). For *o,p*-EDDHA/Fe^{3+} the log Kº (28.72) is lower than that of *o,o*-EDDHA/Fe^{3+} (35.09). Moreover, *p*,*p*-EDDHA is not able to complex Fe in solution in a wide range of pH values. Whereas Fe is bound to 6 donor groups in *o*,*o*-EDDHA/Fe^{3+}, in *o*,*p*-EDDHA/Fe^{3+} only five groups bind the Fe^{3+} ion (see Figure 5-5), allowing the sixth coordination position to be occupied by a water molecule. Other important difference is that *o*,*p*-EDDHA/Fe^{3+} may be easily protonated, and in fact the main species at pH values below 6.30 is the neutral species FeH*o*,*p*-EDDHA (Figure 5-5). Moreover, at pH values above 9.27 the main species is FeH*o*,*p*-$EDDHA^{2-}$. All other *o*,*o*-EDDHA analogues form Fe-chelates with one negative charge, except EDDHSA and EDDCHA, which form Fe-chelates with three negative charges. It should be mentioned that the diagrams shown in Figure 5-4 have been drawn for a normal Cu availability in soils (limited to a maximum of 10 μM of total Cu in soil solution), whereas Cu^{2+} largely affects the stability of *o*,*p*-EDDHA/Fe^{3+} (as well as those of other Fe-chelates at high pH values). In these conditions, *o*,*p*-EDDHA/Fe^{3+} is quite stable, but when the soil has more available Cu^{2+} this ion can displace more Fe^{3+} from the Fe-chelate.

Theoretical chemical speciation is a useful tool to predict the behaviour of Fe-chelates in agronomic conditions prior to their application. However, it should be considered that the main competitors are micronutrients such as Cu, whose availability in the soil is a quite low, and a small variation in the availability of that micronutrient may produce a different Fe-chelate speciation. Moreover, it is difficult to include sorption processes in the chemical speciation studies.

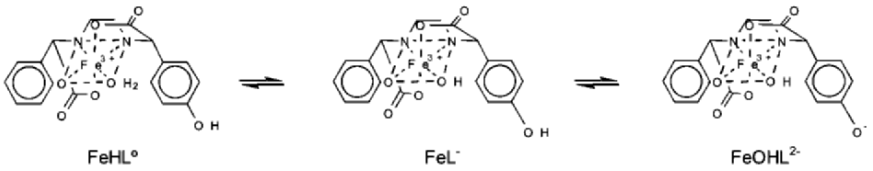

Figure 5-5. Main species of *o,p*-EDDHA/Fe^{3+}.

5. SORPTION OF IRON-CHELATES ON SOIL SURFACES

Since adsorption-desorption reactions tend to be faster than precipitation-dissolution processes, adsorption at solid interfaces can be a dominant factor in regulating micronutrient concentrations in solution (Harter, 1991; Stone, 1977). Adsorption or "fixation" of chelating agents in soils was recognized in early investigations of the agricultural uses of synthetic chelating agents (Wallace and Lunt, 1956; Hill-Cottingham and Lloyd-Jones 1957; Aboulroos *et al.*, 1983; Norvell, 1991). The effectiveness of Fe-chelates as Fe sources and carriers in soil can be severely limited by the adsorption of Fe-chelates or chelating agents in the solid phase (Sánchez-Andreu *et al.*, 1991). The factors affecting adsorption include the type of chelating agent, the metal ion, time, pH, salt concentrations and soil texture (Aboulroos *et al.*, 1983). Wallace *et al.* (1955) added various chelates, including Fe(III)-EDDHA, to soils, and found that after 4 days chelating agent losses in a calcareous loam soil ranged from 7 to 30%, whereas losses in a moderately acidic clay soil ranged from 0 to 51%. Adsorption, rather than degradation, was identified as the cause of the losses. Wallace and Lunt (1956) proposed the clay size fraction of soils as the major adsorbent of chelating agents. As it would be expected, the negatively charged surfaces of clay minerals are not effective in adsorbing the predominately anionic chelate species (Hemwall, 1958), and positively charged sites on Fe oxides and other colloids could be more important. Adsorption of chelating agents by peat was also observed, and losses of *o*,*o*-EDDHA/Fe^{3+} can be relatively large at low pH values (Hemwall, 1958). Negatively charged chelates adsorption by surfaces with pH-dependent charge, such as oxides or peat, decreases with rising pH (Norvell and Lindsay, 1972).

Adsorption of chelates by soil oxides may be similar to what occurs with specific Fe oxides (Norvell, 1991). Adsorption of several anionic ligands by hematite (Fe oxide) showed a degree of specific chemisorption in the binding of these anionic ligands in opposition to the net negative surface charge (Chang *et al.*, 1983).

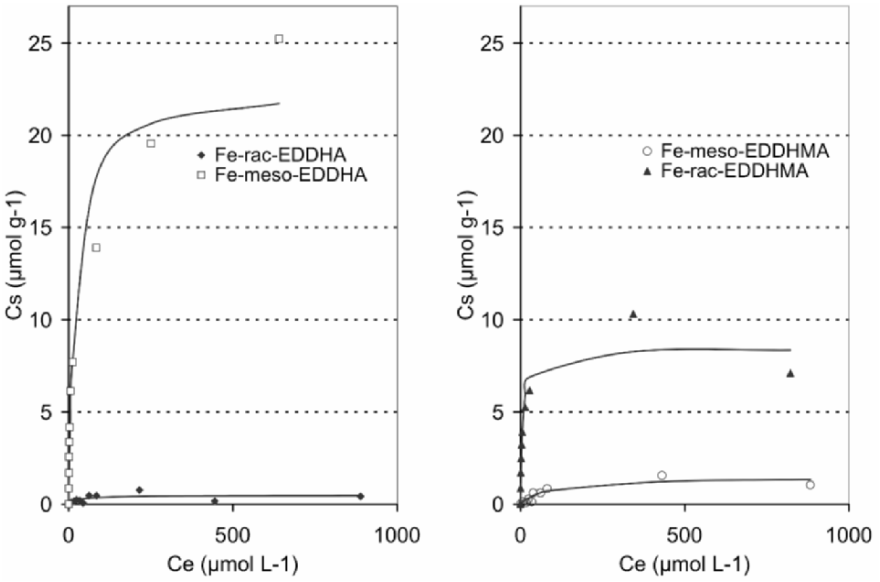

Figure 5-6. Sorption isotherms (T = 25ºC) for the retention of *o,o*-EDDHA/Fe^{3+} and EDDHMA/Fe^{3+} in ferrihydrite at pH around 6.8 (after Hernández-Apaolaza and Lucena, 2001). Cs: Concentration retained; Ce: Concentration in the solution. Lines are the Langmuir fits.

In a recent study (Hernández-Apaolaza and Lucena, 2001), we investigated the sorption of the different diastereoisomers of *o,o*-EDDHA/Fe^{3+} and EDDHMA/Fe^{3+} on a clay, an Fe oxide and an organic material. We concluded that the sorption on Ca-montmorillonite was probably through a Ca bridge, by the formation of an outer sphere complex montmorillonite-Ca-Fe-chelate, since Fe-chelates are negatively charged. This hypothesis was supported by the fact that both *meso* and *racemic* isomers behaved similarly, the proposed mechanism sorption depending on the net charge more than on the internal arrangement of the molecules. *o,o*-EDDHA/Fe^{3+} showed a higher sorption than EDDHMA/Fe^{3+} by the Fe oxide ferrihydrite (see isotherms in Figure 5-6). The *meso-o,o*-EDDHA/Fe^{3+} isomer was highly adsorbed on this oxide, but the *racemic* isomer was not significantly retained by ferrihydrite. In the case of EDDHMA/Fe^{3+} isomers, the *racemic* one was more retained by the oxide, but also a small degree of sorption was

observed for the *meso* isomer. We suggested that in the sorption on ferrihydrite the *o*,*o*-EDDHA/Fe^{3+} and EDDHMA/Fe^{3+} lose some of their links with the Fe^{3+} ion, probably the ones with the carboxyl groups which are less stable, and then formed an electrostatic binding with the ferrihydrite surface.

In this model, the stability of the chelates is an important factor to understand the sorption process. The EDDHMA/Fe^{3+} chelate shows a higher stability than the *o*,*o*-EDDHA/Fe^{3+} (see Figure 5-4). This probably occurs because it is more difficult for EDDHMA/Fe^{3+} than for *o*,*o*-EDDHA/$Fe^{3+}$to open its spatial arrangement, the former chelate being therefore less adsorbed at the surface. For the *o*,*o*-EDDHA/Fe^{3+} isomers, the *meso* is more retained than the *racemic* one, probably due to its lower stability. For the EDDHMA/Fe^{3+} isomers, it was also the less stable one (*racemic*) the more retained. This model would also explain the pH dependence of the sorption on ferrihydrite. Between pH 4 and 8 (below the isoelectric point), both chelates were largely retained by Fe oxide, but over pH 8 the sorption decreased drastically, because the oxide became negatively charged. When EDDHSA/Fe^{3+} was left to react with a synthetic ferrihydrite, the amount of chelate remaining in solution was 98%, whereas for *o*,*o*-EDDHA/Fe^{3+} it was only 66%. EDDHSA/Fe^{3+} has three negative charges, indicating that the sorption is not merely electrostatic. This chelate presents a high stability (see Figure 5-4), supporting that the rupture of the link between Fe and the carboxyl groups is a step previous to the sorption.

In the same study it was found that *o*,*o*-EDDHA/Fe^{3+} retention in peat was also larger than that of EDDHMA/Fe^{3+}, in good agreement with previous findings (Álvarez-Fernández *et al.*, 1997). The most retained isomer of *o*,*o*-EDDHA/Fe^{3+} on the acid peat surface was the *meso* isomer, possibly due to its lower stability constant with respect to the *racemic* one. The mechanism of sorption of *o*,*o*-EDDHA/Fe^{3+} on peat surfaces is not known, but the higher retention of the *meso*- *o*,*o*-EDDHA/Fe^{3+} could suggest an electrostatic process, where Fe^{3+} can be the binding element. Álvarez-Fernández *et al.* (2002b) found that retention of EDDHSA/Fe^{3+} and EDDCHA/Fe^{3+} was negligible in organic materials.

Despite the advances in the knowledge of the sorption processes in soil materials, the adsorption of metal chelates by soils still remains poorly understood. Full understanding of these reactions will always be difficult, because of the complexity of soil surfaces, the continuing changes in chelate speciation, and the concomitant degradation of the chelating agent. Most of the knowledge has been obtained by batch interaction experiments, where global processes are considered. In general, the most stable Fe-chelates are able to maintain 90-100% of the Fe-chelate in solution after short periods of interaction (Álvarez-Fernández *et al.*, 2002b). In fact, EDDHSA/Fe^{3+} and

EDDCHA/Fe^{3+} were generally as efficient as *o,o*-EDDHA/Fe^{3+} and EDDHMA/Fe^{3+} (the most common and effective Fe fertilisers) to maintain Fe in soil solution for short periods. However, the type of chelating agent is a factor that affects the chelated Fe availability in soil solution, since EDDHMA/Fe^{3+} and *o,o*-EDDHA/Fe^{3+} react with oxides and organic matter more extensively than EDDCHA/Fe^{3+} and EDDHSA/Fe^{3+}. Álvarez-Fernández *et al.* (2002b) also found that the non-chelated Fe present in commercial Fe chelates was stable in solution at the typical pH of calcareous soils, but still exhibited an elevated reactivity in soils, mainly with some components such as oxides and organic matter. Recently, we have studied the interaction of *o,p*-EDDHA/Fe^{3+} with calcareous soils (García-Marco *et al.*, 2005) and we observed (Figure 5-7) that higher amounts of this chelate were maintained in the soil solution as compared with EDTA/Fe^{3+}, but the amounts were much lower than those of *o,o*-EDDHA/Fe^{3+}. These results are similar to the predicted by theoretical calculations (see Figure 5-4) but in this case we observed an even larger decrease of *o,p*-EDDHA/Fe^{3+}, possibly due to the sorption of the neutral species Fe-H*o,p*EDDHA^0 of this chelate.

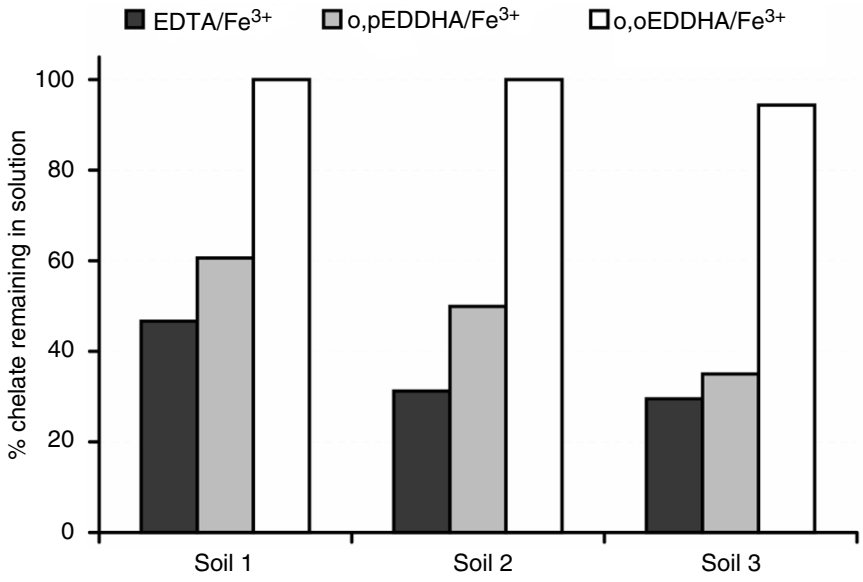

Figure 5-7. Percentage of Fe-chelate remaining in solution after three days of interaction with three calcareous soils.

Recently, García-Mina *et al.* (2003) observed that in a calcareous soil treated with different commercial Fe chelates containing *o,o*-EDDHA, approximately 25-30% of the soluble Fe found at 50 days was not

attributable to *o,o*-EDDHA/Fe^{3+}. They did not determine *o,p*-EDDHA/Fe^{3+}, but suggested the existence of other isomers of EDDHA besides the *o,o* isomer, which could also be available for plant uptake. Similarly, for EDDHSA/Fe^{3+} commercial chelates the amount of soluble Fe not attributable to the monomer EDDHSA/Fe^{3+} was 36-40% of the total Fe, which is in good agreement with the presence of soluble, stable Fe-chelates, possibly consisting in condensation products formed in the industrial synthesis. With respect to the presence of the Fe-chelates in the soluble fraction, they found that both EDTA/Fe^{3+} and DTPA/Fe^{3+} were easily lost from the soil solution, likely due to Ca competition (Figure 5-8). Among the stronger Fe-chelates, EDDHSA/Fe^{3+} and EDDCHA/Fe^{3+} (only the monomers were measured in that study) are those maintained in larger amounts in the soluble fraction, whereas EDDHMA/Fe^{3+} and *o,o*-EDDHA/Fe^{3+} slowly decreased with time.

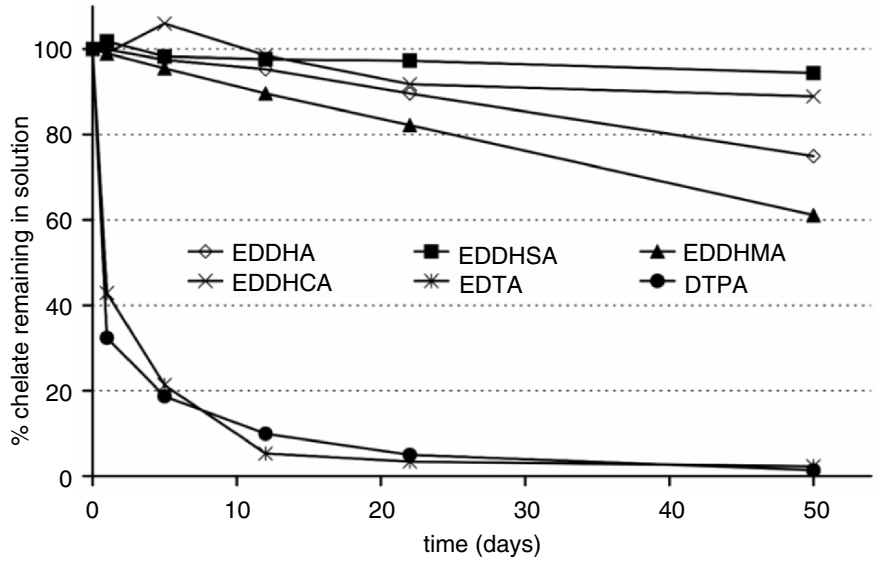

Figure 5-8. Percentage of the Fe-chelate remaining in solution after interaction with a calcareous soil. (Data after García-Mina *et al*., 2003).

While Fe precipitation implies the destruction of the chelate, Fe-chelate sorption could be reversible. In fact, most of the proposed mechanisms are electrostatic. Therefore, sorbed Fe-chelates could serve as a reservoir, acting as slow release fertilizers and increasing the Fe presence in the soil solution in the long term. However, while sorption may provide a longer lasting effect of the Fe-chelates, it also may reduce the fast action normally needed by chlorotic crops.

6. DISSOLUTION OF SOIL NATIVE IRON BY CHELATING AGENTS

When the Fe-chelate releases Fe to the plant, the chelating agent may dissolve native Fe (Figure 5-1), which can be then transported to the plant (Chen and Barak, 1982; Lindsay, 1995; Lucena, 2003). However, the importance of this dissolution process in plant Fe nutrition is not well known. Schwertmann (1991) reviewed the knowledge on the processes of dissolution of Fe oxides, indicating that most of the studies concerning chelating agents have been done for the estimation of the plant-available Fe. The chelating agent may dissolve native Fe present in the solid phases. This is a kinetically controlled process that depends on the chelating agent (Stone, 1977), the nature of the solid phase (Schwertmann 1991; Pérez-Sanz and Lucena 1995; Nowack and Sigg, 1997) and the soil conditions. The rate of dissolution may be the limiting factor for the whole process. It has been hypothesized that kinetics, rather than equilibrium, probably controls the speciation of low stability complexes (such as phytosiderophores; Stone, 1977). Pérez-Sanz and Lucena (1995) studied the kinetic parameters for the Fe dissolution of different Fe oxides in presence of *o*,*o*-EDDHA. Results showed that amorphous $Fe(OH)_3$ had a higher dissolution rate than goethite, maghemite and magnetite. Dissolution rate from amorphous $Fe(OH)_3$ and goethite follows a first order kinetics, while from maghemite and magnetite the order of the reaction was 2. This implies that for the last two the dissolution rate increases with the amount of free *o*,*o*-EDDHA in the media, conversely to what happens for amorphous $Fe(OH)_3$ and goethite. In the same study, the dissolution rate was compared with the Fe nutrition of sunflower grown in hydroponics in presence of both *o*,*o*-EDDHA/Fe^{3+} and different Fe oxides separated from the nutrient solution by a dialysis bag. Authors concluded that Fe uptake was higher when using the Fe oxides with higher dissolution rates. Recently, the dissolution rate of Fe from different soils and Fe oxides by EDTA, *o*,*o*-EDDHA and *o*,*p*-EDDHA has been compared (García-Marco *et al.*, 2005). The amount of Fe solubilized with time fitted well to the equation:

$$Fe(\mu mol/g) = \frac{Fe_{\max} \cdot t}{t_{1/2} + t} \qquad (1)$$

where Fe (μmol/g) is the amount of Fe solubilized by the chelating agent for a time t, Fe_{max} is the maximum amount of Fe that can be dissolved for that chelating agent and $t_{1/2}$ (half time) is the time needed to dissolve half of the Fe_{max}. This last parameter is an index of the rate of dissolution, and it is presented for the materials studied in Table 5-1. The lower the value of $t_{1/2}$,

the faster the dissolution process. It can be observed that the dissolution of *o*,*p*-EDDHA is faster than that of *o*,*o*-EDDHA, and also that EDTA dissolves Fe from all the materials used. However, the maximum amount of Fe that can be dissolved was largest for *o*,*o*-EDDHA and very low for EDTA.

Table 5-1. Kinetic parameters for the dissolution process of Fe oxides and soils in 100 μM chelating agents.

	Maghemite	Goethite	$Fe(OH)_{3amp}$	Soil-1	Soil-2	SCS
Half time ($t_{1/2}$, hours)						
EDTA	10.7	472.1*	3.5	1.91	1.38	1.62
o,o-EDDHA	2.3	91.5	3.1	0.56	1.80	3.14
o,p-EDDHA	1.3	6.1	0.5	0.22	0.22	0.13
Fe_{max} (% of the chelating capacity)						
EDTA	12.3	4.4	1.1	4.1	8.4	2.8
o,o-EDDHA	79.5	101.5	29.4	18.2	47.9	40.0
o,p-EDDHA	59.6	10.6	2.4	5.2	22.4	7.9

Soil-1 and Soil-2: calcareous agricultural soils. SCS: standard of calcareous soils. *Predicted from the experimental data.

7. IRON UPTAKE FROM IRON CHELATES

When an Fe chelate is present in the soil solution, it will refill the Fe^{3+} pool in the root surface as soon as the plant uses it.

$$YFe^{-} \leftrightarrows Y^{4-} + Fe^{3+} \qquad K_s^o = \frac{[YFe^{-}]}{[Y^{4-}][Fe^{3+}]} \tag{2}$$

resulting in:

$$[Fe^{3+}] = \frac{[YFe^{-}]}{[Y^{4-}]K_s^o} \tag{3}$$

Therefore, the higher the Kº and the concentration of free ligand, the lower the concentration of Fe^{3+} allowed by the chelate to be available for the plant. This Fe^{3+} should be reduced before entering the plant. In Strategy I plants the reduction of the Fe-chelate is carried out by an Fe-chelate reductase (FCR) enzyme (Chaney *et al.*, 1972; Bienfait, 1985; Schmidt, 2005), whereas in Strategy II plants (Kawai and Alam, 2005) chelating

agents would compete with phytosiderophores for Fe binding. Since Fe-phytosiderophore complexes are far less stable than synthetic chelates, this may be a problem for the use of Fe-chelates by these plants. After the normal or FCR mediated reduction, Fe^{2+} transport into roots occurs by a plasma membrane transporter (Fox *et al.*, 1996).

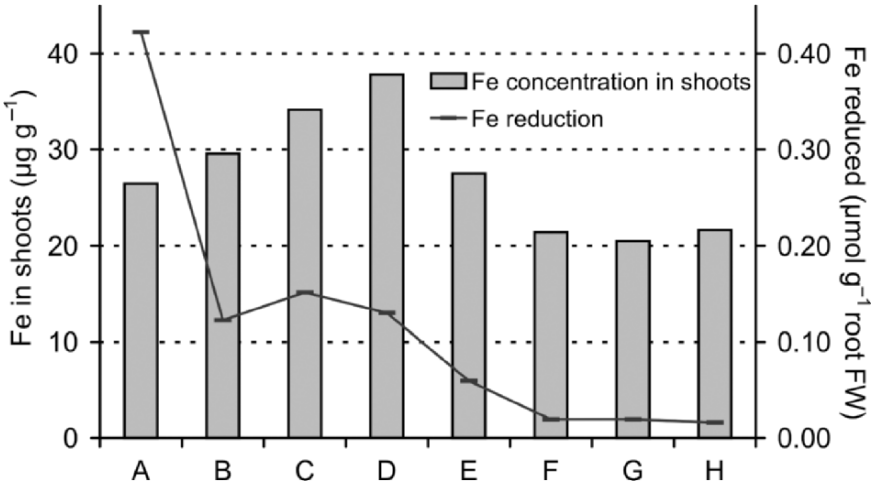

Figure 5-9. Comparison of the Fe reduced by green stressed cucumber plants at pH 7.5 from different Fe-chelates and the concentration of Fe in shoots when the same chelates were used at low concentrations (5 µM) in hydroponic culture (pH 7.5). Chelates are presented in stability sequence (from lower to higher: A: EDTA/Fe^{3+}; B: PDDHA/Fe^{3+} (pentil diamine di(*o*-hydroxyphenilacetic) acid; C: *meso o,o*-EDDHA/Fe^{3+}; D: *o,o*-EDDHA/Fe^{3+}; E: *o,o*-EDDHMA/Fe^{3+}; F: rac *o,o*-EDDHA/Fe^{3+}; G: EDDHSA/Fe^{3+}; and H: HBED/Fe^{3+}). Data re-elaborated after Lucena and Chaney, 2005a and 2005b.

The FCR enzyme is up-regulated by Strategy I plants when Fe is limited in the media (Robinson *et al.*, 1999). The substrates of the FCR are stable, Fe(III)-selective chelates. Chaney (1989) studied the effect of Fe-chelate concentration and nature on the rate of Fe reduction by roots of Fe-deficient peanut plants. No conclusive relation between kinetic parameters (the rate of Fe reduction by roots) and the stability of the Fe-chelates was observed using EDDA (ethylene-diamine-diacetic acid), HEDTA, EDTA and DTPA as chelators. The charge of the species in solution, which varied from 0 to –2 among the four chelates used, could have controlled the rate of reduction. The more stable Fe-chelates were not used in those experiments. FCR has been described to have higher activities at pH values below 6.5 (Moog and Brüggemann, 1995; Susín *et al.*, 1996; Romera *et al.*, 1998), while Fe chlorosis occurs in calcareous soils with bulk soil pH 7.5-8.5. Strategy I also involves up-regulation of H^+ extrusion (Römheld and Marshner, 1986;

Alcántara, 1991; Wei *et al.,* 1997) that may help in a local reduction of the rhizosphere or apoplastic pH, despite the high buffering effect of bicarbonate (Lucena *et al.*, 2000).

Recently, the role of the Fe^{3+}-chelates as substrates of the FCR enzyme, a part of the Fe uptake mechanism in Strategy I plants, has been investigated (Lucena and Chaney, 2005a). It was concluded that a high stability of the Fe-chelate decreases FCR reduction rates in mildly chlorotic cucumber plants, although plants can always take up Fe from very stable Fe-chelates (see also Chaney, 1988). Also, a low Fe concentration in xylem sap was found in plants treated with very stable Fe-chelates. In another experiment (Lucena and Chaney 2005b), cucumber plants were grown with 5 μM concentrations of different chelates. Various Fe nutrition indexes revealed that plants had a better Fe supply when treated with relatively weaker chelates, with the exception of EDTA/Fe^{3+}. A comparison of the results from both experiments is presented in Figure 5-9, showing a similar trend for both parameters in all chelates, excepting EDTA/Fe^{3+}. For chelates other than EDTA/Fe^{3+}, Fe uptake correlates with Fe reduction. Considering the chemical reaction:

$$\text{Fe-Y}^{-} + \text{e}^{-} \leftrightarrows \text{Y}^{4-} + \text{Fe}^{2+} \qquad K = \frac{K_{red}}{K_s^o} = \frac{[Y^{4-}][Fe^{2+}]}{[YFe^{-}][e^{-}]} \qquad (4)$$

where K_{red} is the stability constant for the reduction of the Fe^{3+} to Fe^{2+} ($\log K_{red} = 13.04$, Lindsay, 1979) and K^o_s is the stability constant of the formation of the Fe(III) chelate.

$$\text{then, } \log[Fe^{2+}] = \log\frac{K_{red}}{K_s^o} + \log\left(\frac{[YFe^{-}]}{[Y^{4-}]}\right) - pe \qquad (5)$$

The presence of Fe^{2+} will be therefore controlled by three terms: the first one depends inversely on the stability constant of the Fe(III) complex; the second one depends on the free chelating agent concentration, which is controlled by some side-reactions (protonation and complexation with other metals); and the third is the redox potential that the plant FCR enzyme imposes on the system. Then, the weaker the Fe-chelate, the easier plants can reduce Fe. Although this theoretical scenario could be too simplistic, it is in good agreement with the obtained results (Figure 5-9), where chelates are presented in a sequence from lower (left) to higher (right) stability. We also observed the same behaviour comparing *o*,*o*-EDDHA/Fe^{3+} and

o,*p*-EDDHA/Fe^{3+} While *o*,*o*-EDDHA/Fe^{3+} is more stable than *o*,*p*-EDDHA/Fe^{3+}, the latter can provide Fe faster to the plant (García-Marco *et al.*, 2005).

The EDTA/Fe^{2+} complex is also quite stable, and therefore the Fe^{2+} produced by the FCR activity can be trapped again by free EDTA, competing with BPDS present in the Fe reduction assays. In fact, FCR activities could be twice (at pH 6.0) or 3.5 times (at pH 7.5) faster when EDTA/Fe^{3+} is the substrate than when the source is *o*,*o*-EDDHA/Fe^{3+}. However, the Fe concentration in the xylem sap is double for *o*,*o*-EDDHA/Fe^{3+} than for EDTA/Fe^{3+}, which is in good agreement with the better Fe nutrition provided by *o*,*o*-EDDHA/Fe^{3+} (Figure 5-9).

The determination of the amount of Fe reduced by plants supplied with different Fe-chelates can inform us only about a part of the Fe uptake process. The result of the complete process could be better evaluated from the Fe absorption by the plant. Chaney and Bell (1987) presented data of the Fe reduction from Fe-DTPA and the ^{59}Fe-uptake by stressed peanut plants. In that study, the fraction of Fe reaching the shoots was quite large, close to 100% of the reduced Fe, but the pH values of the uptake assay solutions were approximately 4. The authors observed separate regulation of Fe reduction and uptake rates, with acidification being one of the factors related with the differences in regulation. In our studies (Lucena and Chaney, 2005a and 2005b) Fe uptake was calculated from the concentration of Fe present in the xylem sap, and in both experiments only a small fraction (around 1.1% at pH 6.0 and 0.6% at pH 7.5) of the Fe reduced reached the xylem sap. Discrepancies with the data of Chaney and Bell (1987) may be due by the effect of pH, since we used higher pH values (6.0 or 7.5) than that employed by Chaney and Bell (1987) (around 4.0). Moreover, Chaney and Bell (1987) did not use a pH buffer in the uptake solutions, so that acidification by the roots could likely have increased both the reduction and uptake processes, while in our experiments (Lucena and Chaney, 2005a and 2005b) the final pH was close to the initial one, plant-driven acidification being prevented from affecting both uptake and reduction of Fe.

The Fe^{2+} produced by the FCR that was not absorbed by the plant had to be re- oxidised (or complexed in the case of EDTA). The ultimate electron acceptor for the re-oxidation should be oxygen, but the chelating agents could also catalyse the re-oxidation by favouring the formation of Fe^{3+}-chelate, or could even act as electron carriers (Kurimura *et al.*, 1968). The role of chelating agents in Fe^{2+} re-oxidation under natural conditions of plant growth should receive more consideration, since it may affect the "shuttle effect" that has been proposed for the action of Fe-chelates in soils (Lindsay, 1995).

While Fe reduction and uptake measured in a controlled experiment gave us important information on the factors affecting the efficacy of Fe chelates,

biological experiments including the soil are also needed to have a better knowledge of the effect of Fe-chelates. Several authors have made experiments to compare different chelates (Reed *et al.*, 1988; Hernández-Apaolaza *et al.*, 1995; Álvarez-Fernández *et al.*, 1996), mainly *o*,*o*-EDDHA/Fe^{3+} and EDDHMA/Fe^{3+}, but they have obtained contradictory results, because commercial chelates with unknown Fe-chelate content were used. Recently (Álvarez-Fernández *et al.*, 2005) we were able for the first time to compare chelates with different chelating agents using the same amount of chelated Fe, since a reliable determination method is now available (Lucena *et al.*, 1996 and Hernández-Apaolaza *et al.*, 1997). Álvarez-Fernández *et al.* (2005) compared the application of chelates with the same doses of chelated Fe in three different experiments. We concluded that commercial products containing *o*,*o*-EDDHA/Fe^{3+} and EDDHMA/Fe^{3+} had a similar efficacy to re-green Fe chlorotic plants, both in a soil-less system and in field conditions. However, *o*,*o*-EDDHA/Fe^{3+} presented some advantages, due to its somewhat longer lasting effect. EDDHSA/Fe^{3+} was also effective in correcting Fe chlorosis in pear trees grown on a calcareous soil and in sunflower plants grown in a soil-less system.

8. QUALITY OF COMMERCIAL IRON CHELATES

Commercial products are obtained by carrying out first the industrial synthesis of the chelating agents and then incorporating Fe from inorganic salts. While the first synthesis pathways proposed produced quite pure compounds (Kroll *et al.*, 1957), the industrial synthesis pathways used nowadays (Dexter, 1958; Petree *et al.*, 1978) yield commercial products with quite different purities, and also lead to the presence of by-products. For instance, the condensation product 2,6-di [$CH(COOH)NHCH_2$ $CH_2NHCH(COOH)Ar$] phenol (Ar) hydroxyphenyl (Cremonini *et al.*, 2001) and *o*,*p*-EDDHA (Gómez-Gallego *et al.*, 2002) have been detected in EDDHA commercial products, by using one- and two dimensional nuclear magnetic resonance and ion pair HPLC. Also, main impurities in the EDDHSA/Fe^{3+} and EDDCHA/Fe^{3+} containing products have been studied by HPLC (Álvarez-Fernández *et al.*, 2002a), revealing that unreacted starting materials (*p*-hydroxybenzenesulfonic acid and *p*-hydroxybenzoic acid, respectively) were always present. 1D and 2D NMR experiments (Álvarez-Fernández *et al.*, 2002a) showed that commercial fertilizers based on EDDHMA/Fe^{3+} contained different methyl substitutes of EDDHMA. These findings suggest that current production processes of Fe-chelates used in agriculture are far from providing the very pure compounds that can be obtained with other methods (Kroll *et al.*, 1957; Sierra *et al.*, 2002).

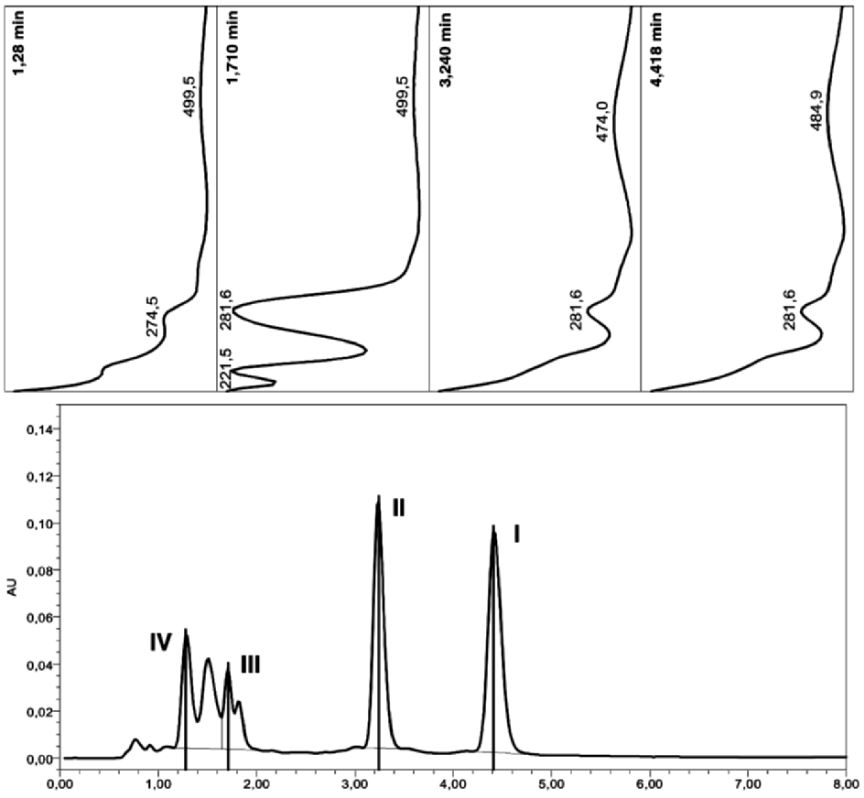

Figure 5-10. A typical chromatogram for the quantification of the *o,o*-EDDHA/Fe^{3+} in commercial products using the method described by Lucena *et al.* (1996). I and II are the geometric isomers (*meso* and *racemic*) of *o,o*-EDHA/Fe^{3+}. III correspond with degradation products of the *p,p*-EDDHA and peak IV is due to the presence of *o,p*-EDDHA/Fe^{3+}. The visible-UV spectra are also included.

Analytical methods have been an important source of information on the quality of the commercial products. Several analysis methods for the quality control of the Fe-chelates have been developed (Boxema, 1979; Barak and Chen, 1987; Deacon *et al.*, 1994: Lucena *et al.*, 1996). A comparative study about the efficacy of the different analysis methods of Fe chelates concluded that high performance liquid chromatography (HPLC) is the most reliable means to quantify the amount of chelated Fe. Among them, the method proposed by Lucena *et al.* (1996) was the simplest and most user-friendly (Hernández-Apaolaza *et al.*, 2000). An example including a chromatogram obtained for a commercial product containing EDDHA/Fe^{3+} is shown in

Figure 5-10. Using this methodology, Hernández-Apaolaza *et al.* (1997, 2000) and Álvarez-Fernández (2000) pointed out that all tested commercial Fe-chelate formulations containing *o*,*o*-EDDHA and EDDHMA and sold in Spain when these studies were carried out, did not meet the declared Fe-chelated content. In fact, using 42 products available before 2000 in Spain, with a declared content of 6% Fe in the form of *o*,*o*-EDDHA/Fe^{3+}, the average chelated Fe content was only 2.59%. Now that a standard analytical method has been approved and is widely used, the actual and declared contents are quite similar in commercial products, purities of the products declaring *o*,*o*-EDDHA/Fe^{3+} being in the range from 3 to 6%.

9. CONCLUSION AND FUTURE OUTLOOK

In this chapter Fe-chelates have been presented as the most efficient, but expensive, fertilizers to correct Fe chlorosis. Their chemical equilibria, the sorption into soil surfaces, the plant acquisition of Fe from chelates and the remobilization of Fe from native forms present in soils seems to play important roles in their mechanism of action, but the quality and effectiveness of commercial products can still be considerably improved.

In the future, research should focus on the synthesis and characterization of new Fe chelating agents, selected not only on the basis of a high stability constant but also according to their capability to deliver Fe to the plant and on the possibility to produce highly pure commercial products at a lower cost.

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Chapter 6

HEAVY METALS COMPETING WITH IRON UNDER CONDITIONS INVOLVING PHYTOREMEDIATION

FERENC FODOR
Department of Plant Physiology and Molecular Plant Biology, Eötvös University, Budapest, H-1518 P.O. Box 120, Hungary. E-mail: ffodor@ludens.elte.hu

Abstract: Heavy metals polluting the environment influence adsorption, uptake and translocation of iron in plants. Toxic metals are usually retained in the roots and there is little accumulation in the shoot, and plants suffer from different levels of stress due in many occasions to insufficient iron supply. However, there are certain plant species or varieties that can accumulate over a thousand times higher concentrations of heavy metals in their shoots as compared to those found in other plant species. These plants have been suggested to be a cheap tool for decontamination of soils, in what is called phytoremediation. Non-accumulator plants may also be used for phytoextraction of heavy metals, by applying chelating agents directly to the soil. Although interactions of heavy metals with iron have been reported in many cases, the specific physiological processes associated with iron deficiency have remained unexplored until the last decade. Since substantial progress has been made in the investigation of iron uptake and translocation using molecular biological techniques, a new horizon has opened in the area of stress physiology. In the present paper, new findings in iron nutrition research are presented in connection with heavy metal uptake and especially hyperaccumulation. Competitive chelation in the rhizosphere, cytoplasm and xylem sap as well as the effect of heavy metals on iron reduction are discussed, while a brief introductory overview on phytoremediation is also provided.

Key words: chelates; heavy metals; hyperaccumulation; iron; phytoremediation

1. INTRODUCTION

Heavy metals have been a major problem in industrial and agricultural areas in the past decades. They still contaminate soils and several of them

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are readily available for plants. Plants may accumulate heavy metals in roots and shoots, causing physiological disturbances in many processes including growth, water relations, photosynthesis, nutrient uptake, allocation and assimilation. Among the nutrients showing interactions with heavy metals, Fe is one of the most frequently concerned in many respects. Heavy metals influence Fe availability and adsorption in the root apoplasm, uptake into root cells, transport to the shoot and utilization in leaves. In turn, Fe deficiency may also modify heavy metal uptake and accumulation.

Recently, alternative methods have been proposed for decontamination of soils that contain heavy metals in elevated concentrations. The methodologies, collectively known as phytoremediation, are based on the capability of some plant species to take up and accumulate certain heavy metals in their tissues, without developing serious stress symptoms. However, the efficiency of metal accumulation greatly depends on environmental factors such as soil properties (e.g. humic substances, pH, etc.), chemical forms of the contaminating metals and the presence of chelating agents. Application of artificial chelating agents to soils in order to induce phytoextraction of heavy metals is now a commercially implemented method. Different types of chelating agents play also a crucial role in plant Fe uptake, therefore affecting both Fe and heavy metal uptake and translocation. This chapter deals with the Fe nutrition of plants as affected by heavy metals under conditions involving phytoremediation, with special emphasis on the role of chelating agents.

2. HEAVY METAL ACCUMULATION IN PLANTS AND PHYTOREMEDIATION

2.1 Heavy metal availability in the soil

Chemical analysis of the soil reveals the concentration of metals but provides little information on their phytoavailability. Even mobile metals are present in the soil in several forms of different mobility, including hydrated ions, soluble organic and inorganic complexes and constituents of floating colloid particles in the soil solution, as exchangeable ions adsorbed on colloid surfaces and in the silicate crystals of the solid phase. There is a dynamic exchange between all these different forms (Marschner, 1995).

Phytoavailable metal concentrations in the rhizosphere can be estimated by solute transfer models that incorporate the metal concentration in the bulk soil solution, the buffering power of the soil, the diffusion coefficient for the metal, the water movement, the size and morphology of roots and the rate of

entry of metals into the roots (Whiting *et al.*, 2003). Other soil properties may also influence the phytoavailable fraction of heavy metals. Such properties are pH, organic matter content, clay particles, moisture content and soil type (Alloway, 1995). For instance, it was shown that spinach (*Spinacia oleracea*), a species capable of accumulating large amounts of heavy metals, took up lower amounts of metals from a clay than from a sandy soil, even at similar total element contents (Naidu *et al.*, 2003).

There are differences between heavy metals concerning their phyto-availability. Lead and Cu have a high affinity for organic soil constituents, although they may also bind to colloid particles. Zinc has a much lower affinity for soil organic particles than Cu. The phytoavailable fraction of Zn corresponds to its water-soluble and exchangeable forms, which constitute a small portion as compared to the total Zn content of the soil. Cadmium is a constituent of fertilizers and sewage sludge applied to agricultural areas, and is highly mobile and available for plants (Bolan *et al.*, 2003; McBride, 1989; Bell *et al.*, 1991b; Luo and Christie, 1998; Barak and Helmke, 1993; Kabata-Pendias, 2001).

One of the most important properties affecting heavy metal availability is pH. Generally, heavy metals are more soluble at low pH, and thus factors increasing pH may decrease phytoavailability of metals. Availability of Pb is decreased by phosphates by increasing the inorganically bound fraction and pH (Bolan *et al*., 2003). Lime application to soils also increases pH, counterbalancing Cu release from sewage sludge (Merry *et al.*, 1986), but it often results in lime-chlorosis by decreasing Fe availability (Nikolic and Römheld, 2002). On the contrary, Fe deficiency in most dicots causes an increased activity of root plasmalemma H^+-ATPases, decreasing rizosphere pH and increasing the solubility of Fe and other heavy metals (Marschner *et al.*, 1986).

Cieslinski *et al.* (1997) investigated the low molecular weight organic acids released by five cultivars of a mono- and a dicotyledonous plant (*Triticum turgidum* and *Linum usitatissimum*), and detected oxalic, malonic, fumaric, succinic, acetic, malic, citric and tartaric acids in the root exudates. All these compounds decrease the pH of the rhizosphere, thereby increasing the solubility of most heavy metals, but, on the other hand, they may also bind competitively free metal ions. It has been shown that oxalate secreted from the roots of Pb-tolerant rice varieties may reduce the bioavailability of Pb through precipitation (Yang *et al.*, 2000).

Graminaceous plants (following Strategy II in Fe uptake) release phytosiderophores (e.g. mugineic acid and avenic acid), which are especially powerful in complexing Fe^{3+}, but may also mobilize Mn^{2+}, Zn^{2+} and Cu^{2+} (Römheld, 1991). Siderophores released by soil microorganisms (e.g.

ferrichrome and ferrioxamine B) may increase the soluble fraction of metals in the soil and may also promote their uptake by plants (Crowley *et al.*, 1987).

Since Fe chlorosis is a serious agricultural problem in certain areas, many procedures have been developed to increase soil Fe availability. One of these is the application to the soil of synthetic chelating agents (García-Marco *et al.*, 2003). As chelating agents bind Fe and other heavy metals, they may be used for increasing the solubility and phytoavailability of toxic metals for decontamination purposes (see below). The application of CDTA or DTPA caused a shift in the distribution of Pb in the soil from more recalcitrant to more soluble forms, greatly increasing the leaching of this metal (Cooper *et al.*, 1999). EDTA, which has a high stability constant with Fe^{3+}, also enhances Pb solubility (Blaylock *et al.*, 1997; Huang *et al.*, 1997). However, EDTA application to soils may result in long-term solubilization of heavy metals. For instance, in a study with two metal contaminated soils, Cu-EDTA and Cd-EDTA complexes were still found in soil pore water 5 months after the application of the chelating agent (McGrath *et al.*, 2002). Metal-EDTA complexes with relatively high stability constants (i.e. Cu-, Fe-, Pb- and Zn-EDTA) are degraded more slowly than those with lower stability constants (i.e. Ca-, Mg- and Mn-EDTA) (Satroutdinov *et al.*, 2000).

2.2 Heavy metal accumulation in plant tissues

Heavy metals contaminating soils in elevated concentrations can be essential (Mn, Zn, Cu and Ni) or non-essential (Cd, Pb, Hg, Cr, etc.) for plants. As a first step for ion uptake, all these metals are adsorbed in the root apoplasm as divalent or trivalent cationic forms, replacing single charged cations and Ca^{2+} at cell wall binding sites. Heavy metal concentrations tend to increase in the apoplasmic space as compared to the average concentration in the soil or the rhizosphere, usually inducing high root metal concentrations, as it has been reported in many studies. Another possibility is that certain heavy metals may precipitate on root adsorbing surfaces. For example, in ragweed (*Ambrosia artemisiifolia*) Pb was suggested to accumulate in the apoplast as Pb-P (Huang and Cunningham, 1996).

In accumulator plant species, metals are concentrated in above-ground organs in low to high metal soil concentrations (Baker, 1981). Accumulation may reach 100-1000 times the concentration of metals as compared to non-accumulating plants. The term hyperaccumulation was introduced by Brooks *et al.* (1977), and criteria for defining Ni, Co, Cu, Pb, Se, Zn, Mn and Cd accumulators have been set (Baker *et al.*, 2000; Brooks 1998). Hyper-accumulator species may also be distinguished from other metal accumulating ones by shoot/root metal concentration ratios exceeding 1 (McGrath *et al.*, 2002).

The sink for accumulation may be different in different plant species due to variable anatomical features. The major metal accumulation in tissues usually occurs in trichomes and leaf cells, although metals may bind to cell wall binding sites in the xylem, which may reduce their transport to the leaf, and may also accumulate unspecifically in evaporating surfaces. For example, Zn is accumulated mainly in leaf epidermal cells in *Thlaspi caerulescens* (Küpper *et al.*, 1999; Vázquez *et al.*, 1992). Zinc and Cd may accumulate in the basal compartment of trichomes of *Arabidopsis halleri*. The epidermal cells in this plant are very small and as the accumulation increases the metals can be found in mesophyll cells (Küpper *et al.*, 2000; Zhao *et al.*, 2000). Preferential Cd accumulation in leaf trichomes was also found in Indian mustard (*Brassica juncea*) (Salt *et al.*, 1995).

The physiology behind the accumulation of heavy metals in the shoot involves xylem loading and translocation, leaf cell uptake and storage in high concentrations. The possible interactions of heavy metals and Fe in these processes are discussed below.

2.3 Phytoremediation

Most plant species respond to elevated heavy metal concentrations with stress reactions. However, hyperaccumulator species can grow in habitats heavily contaminated with different metals, storing them in their shoots in amounts higher than 1% of dry biomass, without showing stress symptoms. This observation led to the idea that pollutants can be removed from soils by plants (Chaney, 1983), which has already developed into a cost-effective remediation solution for thousands of contaminated sites. Soil phytoremediation technology for metals is currently divided in two areas, the first one called phytoextraction (phytovolatilization for some metals), which is applied to remove metals, and the second one called phytostabilization, which is applied to reduce bioavailability of pollutants or stabilize contaminated land by reducing erosion. These technologies have been applied successfully for Pb, Cd, As, Cr, Hg, Cu, Zn and certain radionuclides (for review see Salt *et al.*, 1998; McGrath *et al.*, 2002; Singh *et al.*, 2003).

Phytoextraction is based on two basic strategies: (i) continuous phytoextraction and (ii) induced phytoextraction. Continuous phytoextraction is based on hyperaccumulator plant species that can grow and complete their life cycles on metal contaminated sites (Baker and Brooks, 1989). A few examples of this type of plants are *Thlaspi caerulescens* and *Arabidopsis halleri*, which can accumulate Zn, *Alyssum bertolonii* for Ni and several *Astragalus* species for Se (McGrath *et al.*, 2002). All these are slow growing and low biomass plant species, but research aimed at finding more

naturally growing plant species and improving existing genotypes may yield useful plants.

Effective and fast decontamination of soils require fast growing plants with large biomass and capable of hyperaccumulation of metals. Although there is evidence for the existence of this kind of species, such as the Ni hyperaccumulator *Berkheya coddii* from South Africa (Salt *et al.*, 1998), most large biomass plants are sensitive to heavy metal stress. For remediation purposes the crucial condition is the total extraction of metals, whereas the physiological state of the plant material is less important, provided plant harvest and removal is possible. For this reason, the observation that application of synthetic chelates enhances bioavailability and uptake of heavy metals has led to the concept of induced phyto-extraction. The procedure involves planting and cultivating in heavy metal contaminated sites plant species with a large biomass, such as Indian mustard, sunflower or corn. When the phytomass is large enough to accumulate a substantial amount of the metals, a specific chelating agent is applied to the soil and plants are harvested after a short uptake period (Salt *et al.*, 1998; McGrath *et al.*, 2002). This procedure was applied to mobilize Pb, which is otherwise fairly insoluble in most soils due to binding to organic matter and precipitation as phosphates (see above). Growing *Brassica juncea* in Pb contaminated sites and then applying EDTA resulted in an efficient solubilization of Pb, and in the uptake and accumulation of the Pb-EDTA complex by the plants. However, the fact that EDTA is not a specific chelator for Pb leads to an interaction with Fe, further complicated by the influence of plant roots. This type of interactions will be discussed in the next section.

3. INTERACTION OF HEAVY METALS WITH IRON DURING THEIR UPTAKE AND TRANSLOCATION

3.1 Interaction in the rhizosphere

Organic ligands bind metal ions with different stability constants. In the soil solution in the rhizosphere, metals can either form this kind of complexes or precipitate with inorganic anions. Knowing the stability constants of complexes (Table 6-1) and the pH of the nutrient solution, it is possible to theoretically predict which metals and ligands will form complexes and which will precipitate, using specialized software developed for this purpose (MinteqA2, Allison *et al.*, 1991; Geochem-PC, Parker *et al.*, 1995). Any software, however, may fail to give a reliable prediction in soils,

where many different metals and ligands are present in the rhizosphere and physico-chemical conditions (e.g. adsorptive surfaces) are very diverse.

Undoubtedly, heavy metals compete with Fe for organic ligands. Although Fe^{3+} forms the most stable chelates as compared with divalent heavy metal ions, after reduction of Fe^{3+}-chelates at the outer surface of the root cell plasmalemma in dicotyledonous plants the released free chelating agents may chelate other metals such as Pb^{2+} or Cd^{2+} if no more free Fe^{3+} ions are available (Srivastava and Appenroth, 1995). The competition would also depend on the stoichiometry of competing ions.

Table 6-1. Stability of some metal chelate complexes presented as logK for the equation $Me^{n+} + L^{m-} = MeL^{(m-n)-}$. (L: EDTA, ethylenediamintetraacetic acid; citrate; MA, mugineic acid; DMA, deoxymugineic acid; NA, nicotianamine).

Metal	EDTA[1]	Citrate[1]	MA[3]	DMA	NA
Fe^{3+}	25.1	11.5	17.7	18.1[2]	20.6[2]
Fe^{2+}	14.3	4.4	10.1	10.4[3]	12.8[4]
Zn^{2+}	16.5	5.0	12.7	12.8[3]	15.4[4]
Cu^{2+}	18.8	5.9	18.1	18.7[3]	
Mn^{2+}	13.9	4.2	8.3	8.3[3]	
Ni^{2+}	18.6	5.4	14.9	14.8[3]	
Cd^{2+}	16.5	3.8			
Pb^{2+}	18.0	4.1			

[1]Smith and Martell, 1989, [2]von Wirén *et al.*, 1999, [3]Murakami *et al.*, 1989, [4]Anderegg and Ripperger, 1989.

Metal ions are first adsorbed by the root/cell wall carbohydrate network. Its pectin constituents, glucuronic and galacturonic acids, have diffusible protons that can be easily exchanged by metal ions. The adsorption of metals on these binding sites is of the same nature as complexation with soluble organic acids. However, no specific stability constant has been determined for metal binding at the cell wall, due to difficulties originated from their complex nature. In cucumber treated with 10 μM $Pb(NO_3)_2$ and supplied with the same concentration of Fe-citrate or Fe-EDTA, the relative stability of cell wall-Pb bonds was determined by bleaching experiments with Na_2EDTA and citric acid, respectively. It was found that the stability order was Pb-citrate < Pb-cell wall < Pb-EDTA (Varga *et al.*, 1997). Iron chelates having similar or higher stability as Pb-EDTA (Table 6-1) usually tend to stay in the solution instead of being adsorbed at the cell wall, resulting in very low Fe concentrations in the apoplast. In cucumber roots, Fe-accumulation was approximately 3 times higher with Fe-citrate than with Fe-EDTA, both in control and Cd-treated plants (Fodor *et al.*, 1996). Iron concentrations may increase in the root surfaces in unbuffered solutions even when chelates such as Fe-EDTA are applied, as a consequence of Fe precipitation due to low chelate stability above pH 6.5 (Strasser *et al.*, 1999).

The real amount of metals adsorbed either specifically or unspecifically in the root apoplast is not easy (if at all possible) to be determined. So far, the only apoplastic Fe determination methods generally accepted are those based on the reduction of Fe^{3+} in N_2 atmosphere by sodium dithionite and subsequent complexation with α,α-bipyridyl (first proposed by Bienfait *et al.*, 1985; Becker *et al.*, 1992; Nikolic *et al.*, 2000). This procedure is believed to remove reliably all apoplastic Fe. Besides, strongly and weakly bound Fe fractions can be distinguished by using separately a cell wall isolation protocol and an infiltration centrifugation technique (Nikolic and Römheld, 2003). Although the procedure of Bienfait *et al.* (1985) was initially designed for roots, it may be generalized that this method solubilizes only a weakly bound fraction of Fe, which is about twice as large as the strongly bound fraction. However, it is unclear if the strongly bound fraction of Fe, and perhaps those of other heavy metals, has any significance concerning their metabolic utilization.

There is a lack of information on the apoplastic concentrations of other heavy metals, although some attempts have been made to have an estimate. An example is the use of sodium EDTA for the removal of metals from extracellular spaces (Varga *et al.*, 2000, 2002). Studies with Cd-, Ni- and Pb-treated plants revealed that the majority of Pb and Fe accumulated in the apoplasm (more than 70% and 76%, respectively), whereas more than 65% of Cd, 85% of Ni and 67% of Mn (the latter only in Pb treated plants) were not removable by EDTA. Heavy metals applied at the same concentration as Fe (10 μM) increased the total and apoplasmic concentration of Fe in the roots. Results, however, depended on the form of Fe used. Both apoplasmic Pb and Fe increased in Cd-, Ni- and Pb-treated plants when Fe-citrate was supplied as compared to Fe-EDTA (Varga *et al.*, 2002). Although the authors did not carry out a validation for the removal procedure and the scavenging of metals from the apoplasm may not be complete, the results can be well interpreted. Comparing the stability of complexes formed by EDTA and the above metals (see Table 6-1) it is clear that Fe can be removed most efficiently, followed by Cu, Ni and Pb. However, Ni was retained in the roots, and may be strongly bound or absorbed into the cytosol. These data suggest that chelating agents may substantially influence metal adsorption patterns in root cell walls, but for their accumulation it may be more important how they are taken up into the cytosol.

Complexation with chelates not only improves solubility of metal ions, but also ensures their mobility. However, the complexes must reach the root cell plasmalemma transport proteins as a prerequisite to uptake, and the cell wall matrix is a natural filter. Apart from the cation exchange function of pectin chains, that reduces the intrusion of cations, the network of filaments contains pores, and the interfibrillar and intermicellar spaces spatially limit

the free movement of particles larger than hydrated ions or low molecular weight organic solutes. The size of metal chelates is beyond this limit (Marschner, 1995). However, there may be substantial differences in pore size in plant species. How do the complexes reach the plasmalemma and the transporter proteins? Despite this open question, Fe-chelates are very efficient in supplying plants with Fe, and complexes of other heavy metals have been also shown to facilitate their accumulation in the shoot.

3.2 Iron reduction and uptake as affected by other metals

Dicotyledonous and non-graminaceous monocotyledonous species (Strategy I plants) acquire Fe through the action of plasma membrane bound enzymes, such as that already cloned from *Arabidopsis thaliana* (*FRO2,* Robinson *et al.*, 1999). This obligatory step of ferric-chelate reduction prior to Fe uptake (Chaney *et al.*, 1972) is differentially sensitive to heavy metals. In Fe deficient cucumber plants, Pb^{2+} and Mn^{2+} did not affect the induction of root ferric-chelate reduction, while $Cd^{2+}$and Cu^{2+} severely inhibited it at a concentration of 5 μM. Inhibition was also observed after addition of 20 μM Ni^{2+}, 40 μM Mo^{6+} and 100 μM Zn^{2+} (Alcántara *et al.*, 1994). The reduction process itself can be inhibited by Cu^{2+}, Ni^{2+} and Mn^{4+} (Alcántara *et al.*, 1994; Lucena *et al.*, 2003), whereas Cd and Pb (except for Pb at a concentration as high as 2 mM) did not affect it (Chang *et al.*, 2003). When a short term (30-60 min) Cd and Pb treatment was applied to intact Fe deficient sugar beet roots, both Cd and Pb decreased reductase activity, and the effect of free ionic Cd^{2+} was stronger than that of Cd-EDTA or Pb-EDTA (Chang *et al.*, 2003). Heavy metals may interfere with Fe-chelate reduction by several mechanisms as suggested by Lucena *et al.* (2003): (i) redox-active metal ions such as Cu^{2+} and Mn^{2+} could be reduced by the ferric chelate reductase and consequently they may compete with Fe^{3+}-chelates; (ii) metal ions may affect the reductase enzyme, and (iii) metal ions may have an indirect effect on ferric chelate reduction through cell metabolism e.g. by affecting membrane permeability. The case of Cu^{2+} is a special one, because it can be reduced at the plant root plasma membrane prior to its uptake, unlike other metals. There are some pieces of evidence supporting the hypothesis that Fe^{3+} and Cu^{2+} can be reduced by the same enzyme, although it seems that the Cu substrate for reduction may be the free ionic form Cu^{2+} (Lucena *et al.*, 2003).

Another emerging problem is the fate of the product of the reductase action, since Fe^{2+} is highly reactive and its chelates are far less stable than those of Fe^{3+} (Table 6-1). In the green algae *Chlamydomonas reinhardtii*, a copper containing ferroxidase enzyme was identified. This multicopper

oxidase was suggested to have a role in Fe acquisition by reoxidizing Fe^{2+} before uptake to cells (Herbik *et al.*, 2002). Algae fail to exhibit a high affinity Fe uptake in Cu-depleted media, demonstrating the requirement of Cu for the enzyme function. Furthermore, a laccase-like multicopper oxidase (LMCO), also exhibiting ferroxidase activity, was identified in transgenic tobacco cells expressing an LMCO cDNA cloned from *Liriodendron tulipifera* (Hoopes and Dean, 2004). These findings may have implications for the involvement of Cu in high affinity Fe uptake by Strategy I plants.

Several early and recent studies reported that Fe deficiency can be caused by an excess of heavy metals such as Cd (Fodor *et al.*, 1996; Siedlecka and Krupa, 1999) and in some cases Pb (Wallace *et al.*, 1992). One of the best known signs of Fe deficiency is an increase in the activity of the root reductase enzyme. In Fe sufficient sugar beet plants treated with 10 or 50 μM Cd^{2+}, root tip reductase enzyme activity was increased 3.3-4.1-fold. Moreover, Cd-EDTA and also Pb-EDTA (though the latter one at 2 mM concentration) also increased the reduction 2.3-3-fold (Larbi *et al.*, 2002; Chang *et al.*, 2003). These reductase activity increases were accompanied by other physiological changes, including decreases in major photosynthetic pigments, increases in ratios of protective carotenoids to Chls, de-epoxidation of xanthophylls cycle pigments and changes in Chl fluorescence parameters at moderate (10 μM) Cd treatments, but were not associated with low shoot Fe levels, conversely to what has been reported elsewhere (Fodor *et al.*, 1996). Higher Cd concentrations (50 μM) severely reduced growth along with Fe demand of the tissues, and consequently physiological parameters usually associated with Fe deficiency, other than reductase activity, were less marked (Larbi *et al.*, 2002). The controversial data reporting an inhibitory Cd effect on the induction of reductase activity in Fe deficient cucumber and a stimulatory Cd effect in Fe sufficient sugar beet are difficult to explain. One possible explanation might be a differential expression of FRO2 in connection with Fe deficiency induced transfer cell formation (Landsberg, 1995), an issue not studied in Cd-treated sugar beet (Larbi *et al.*, 2002). The inhibitory effect of Pb on Fe-chelate reductase activity was accompanied by a decrease in fine root Fe content and shoot Cu content, and thus an incipient Cu deficiency could explain the otherwise not significant effect (Larbi *et al.*, 2002). Further research is needed, with special emphasis in hyperacumulator species and chelating agents applied in induced phytoremediation.

Generally, an excess of heavy metals, either non-essential or essential, may cause Fe deficiency in plants grown in different types of soils or nutrient solutions (Wallace *et al.*, 1992). However, most of the early observations did not provide evidence for a direct inhibition of Fe uptake in root cells. Recently, Cd has received much attention since it specifically

reduces Fe uptake and/or translocation (Fodor *et al.*, 1996). However, similar effects of other metals such as Ni in cabbage (Yang *et al.*, 1996; Pandey and Sharma, 2002) or Pb in cucumber (only when supplied together with Fe-citrate) (Varga *et al.*, 2002) have been also reported in the literature.

The Fe nutrition status of plants may significantly modify heavy metal uptake. Experiments on the interaction of Fe supply with Cd uptake revealed that a moderate excess of Fe in nutrient solution could have a detoxifying effect. An overdose of Fe decreases Cd accumulation in chloroplasts of primary bean leaves treated with Cd in the nutrient solution (Siedlecka and Krupa, 1996). On the other hand, decreasing the supply of Fe may increase heavy metal uptake. In the Ganges ecotype of the hyperaccumulator *Thlaspi caerulescens* (extremely efficient in Cd accumulation), Fe deficiency increased the V_{max} of Cd influx 3-fold as compared to Fe-sufficient plants, whereas Zn influx was not influenced by the Fe status (Lombi *et al.*, 2002). In Fe-deficient *Commelina communis* an increased uptake of Cu was observed, probably associated with an increase in root ferric chelate reductase activity and not to increases in H^+ release (Chen *et al.*, 2004).

In recent years, substantial progress has been made concerning the genetic background of heavy metal uptake. In Strategy I plants, the high affinity transporter gene *IRT1* has been identified by functional complementation of the yeast *fet3fet4* Fe uptake double mutant in *Arabidopsis thaliana*. Orthologs of *IRT1* have also been identified in tomato and pea (Eide *et al.*, 1996; Eckhardt *et al.*, 2001; Cohen *et al.*, 1998). An *IRT1* homolog has been cloned from rice showing the presence of this transport route in Strategy II plants (Bughio *et al.*, 2002). There is evidence that IRT1 may facilitate also Zn, Mn, Co and Cd transport (Cohen *et al.*, 1998; Vert *et al.*, 2002; Conolly *et al.*, 2002). The expression of genes encoding Fe^{2+} uptake under Fe deficiency is up-regulated in the Ganges ecotype of *Thaspi caerulescens* (Lombi *et al.*, 2002), and this may be related to the enhanced Cd uptake.

Formerly, Cd uptake was demonstrated to occur also by other transport pathways. In *Thlaspi caerulescens* two Zn transporters, ZNT1 and ZNT2 (belonging to the ZIP family: zinc-regulated transporter/Fe-regulated transporter like proteins) were identified. Through functional complementation in yeast, *ZNT1* was shown to mediate high-affinity uptake of Zn^{2+} as well as low-affinity uptake of Cd^{2+} (Pence *et al.*, 2000; Maser *et al.*, 2001). However, in the Ganges ecotype of *Thlaspi cearulescens* Cd accumulation was not connected to the Zn transport pathway (Lombi *et al.*, 2002). In contrast, in the low Cd-accumulating ecotype (Prayon), Cd uptake was suggested to be partly mediated via Ca channels or transporters for Zn and Mn (Zhao *et al.*, 2002). Patch-clamp studies with *Vicia faba* guard cell

protoplasts also showed that Ca^{2+} channels were permeable to Cd^{2+} (Perfus-Barbeoch *et al.*, 2002).

Another family of transporter proteins was identified in *Arabidopsis* with possible functions in Fe homeostasis. *AtNRAMP1, AtNRAMP2* and *AtNRAMP4* (natural resistance-associated macrophage proteins) are up-regulated under Fe deficiency and may function in the transport of Fe and other metals such as Cd and Mn (Curie *et al.*, 2000; Thomine *et al.*, 2000). The fact that *OsNRAMP* was cloned from rice implies that the NRAMP-type transporters have common basic functions in plants (Thomine *et al.*, 2003).

Plant uptake mechanisms for metals other than Fe are much less known, although there are potentially useful data obtained with yeast. The high affinity Fe uptake system in yeast has many similarities with plants (reviewed by Eide, 1998). The yeast low affinity system is catalysed by the proteins FET4p (Dix *et al.*, 1994), Smf1p and Smf2p (Liu *et al.*, 1997). These are single proteins with broad metal specificity and preference for Fe^{2+} over Fe^{3+}. FET4 also acts as a low affinity Cu transporter (Hassett *et al.*, 2000). High affinity Cu transporters such as COPT1-5 have been identified in *Arabidopsis*, although their subcellular location has not been determined yet (Sancenon *et al.*, 2003).

Iron uptake in Strategy II plants is mediated by a Fe^{3+}-phytosiderophore transporter, which has been cloned from maize and named *YS1* (yellow stripe 1). Interestingly, eight *YSL* (yellow stripe 1-like) genes have been recently identified in *Arabidopsis*, and although the function of these genes is not known yet, they might have a role different to that played by *YS1* in Strategy I plants (Curie *et al.*, 2001). Transport of Zn through the phytosiderophore pathway has also been demonstrated in maize and wheat (von Wirén *et al.*, 1996; Rengel *et al.*, 1998). As in Strategy I plants, the Fe nutritional status may influence heavy metal uptake in Strategy II plants. In barley (*Hordeum vulgare*), Fe and Cd were mostly antagonistic, and the effects were not additive at least at Fe concentrations larger than 100 μM (i.e. ten times higher than normal supply), as shown by shoot growth, catalase activities and transcript levels of dehydroascorbate reductase, catalase and glutathion peroxidase. When Fe supply was increased from the normal values to 250 μM, leaving Cd unchanged at 25 μM, there was a small decrease in root Cd content and the root-shoot translocation of Cd was strongly suppressed. These data suggest that Cd can be possibly taken up *via* the phytosiderophore pathway (Sharma *et al.*, 2004).

The fact that metals present in the rhizosphere in toxic concentrations can readily form complexes, not only with natural but also with synthetic chelating agents, increases the possibility that plants can accumulate metals in competition with Fe in a concentration dependent manner, as mentioned above. Stimulation of Cd uptake in Fe-deficient plants grown in soils may be

due to the release of complexing substances (Awad and Römheld, 2000). In Fe-deficient cucumber plants grown in nutrient solution with Fe-EDTA, Fe uptake was greatly increased when plants were exposed to Pb or Cd. However, Fe uptake from Fe-citrate was much higher as compared with Fe-EDTA, and it was increased by Cd and slightly decreased by Pb (Fodor *et al.*, 1996). At a normal Fe supply, basically similar changes were observed in Fe uptake, but Cd caused a much larger increase in root Fe content in case of Fe-citrate (Varga *et al.*, 1999; Varga *et al.*, 2002). At low concentrations (e.g. 10 μM) EDTA (especially as Fe-complex), may exert a protective effect by preventing or decreasing Cd accumulation through complexation outside the root cells (Srivastava and Appenroth, 1995). The simultaneous application of Fe-citrate and Cd did not change Cd accumulation in the root and shoot (Senden *et al.* 1995).

The accumulation of heavy metals increases dramatically at elevated concentrations of chelating agents, as it was shown for Pb in *Brassica juncea* (Vassil *et al.*, 1998). Under these conditions, plant tissues suffer concentration increases not only for heavy metals, but also for the chelating agents. Application of 0.25 mM EDTA for the mobilization of Pb resulted in reductions in transpiration rate and shoot water content correlated with the presence of free protonated EDTA in the nutrient solution, suggesting that protonated EDTA can be more phytotoxic than the Pb-EDTA complex.

The physiological and molecular background behind the uptake of metal-chelates and free chelating agents is not clarified to date. The uptake and breakdown of EDTA by plants was demonstrated in early studies (Hill-Cottingham and Lloyd-Jones 1961, 1965). In *Brassica junicea* the kinetics of Pb and EDTA accumulation were found to be biphasic, suggesting that a threshold concentration of EDTA (0.25 mM) is required to induce EDTA or Pb-EDTA hyperaccumulation in the shoot (Vassil *et al.*, 1998). The same was found earlier for Fe-EDDHA, the threshold level being 1 mM in different species (Jeffreys and Wallace, 1968). It was hypothesized that synthetic chelates at the threshold concentration may induce metal-chelate accumulation by removing stabilizing Zn^{2+} and Ca^{2+} from the plasma membrane, leading to rapid equilibration of the hydroponic or soil solution with the xylem sap (Vassil *et al.*, 1998). Thus, the concentration-dependent transition for the competition of Fe and heavy metals would be limited by the threshold concentration of chelating agents.

3.3 Iron homeostasis and translocation as affected by other metals

The array of Fe-regulated transporters suggests a complex network of intra- and inter-cellular Fe trafficking, leading to the homeostasis of Fe

according to the needs of the plant (Schmidt, 2003). As mentioned above, little is known about the molecular mechanisms of the interactions between heavy metals and Fe during their influx to the cytoplasm, but even less data concerning heavy metal interaction with Fe regulation inside the cell are available. It was found that under Fe-starvation the disruption of *AtNRAMP3* led to increased accumulation of Mn and Zn in the roots, whereas its over expression down-regulates Mn accumulation and also the expression of IRT1 and FRO2 responsible for high affinity Fe uptake (Thomine *et al.*, 2003). The authors suggested that AtNRAMP3 localized in the vacuolar membrane influences metal accumulation and Fe uptake by mobilizing vacuolar metal pools (Fe, Cd and other metals) to the cytosol.

The intracellular level of Fe and other heavy metals are by all means strictly regulated, and different substances are involved in the process. Nicotianamine (NA), a non-proteogenic amino acid ubiquitous in higher plants, seems to be the principal chelator of Fe within the cell when Fe is not bound to target molecules such as heme or stored as phytoferritin (Hell and Stephan, 2003). Nicotianamine may function in keeping Fe soluble and available as well as preventing Fenton reactions, which lead to oxygen radical formation when free Fe^{2+} ions are present (von Wirén *et al.*, 1999). Organic acids and amino acids are also available chelating agents for Fe, although the stability constants of those complexes are high enough only for Fe^{3+} (see Table 6-1 for citrate).

Phytochelatins (PCs) are Cys-rich molecules with the structure $(\gamma\text{–Glu–Cys})_n\text{–Gly}$, where n is the number of repetition of the unit (usually 2-11). Phytochelatins chelate heavy metals, such as Cu, Cd and Hg, which have high affinity with the thiolic groups of Cys, and play a crucial role for their sequestration into the vacuole. These compounds are synthesized from glutathione by the enzyme PC synthase. This mechanism may be the most significant response of plant cells to heavy metal toxicity, because the PC synthase is induced promptly and greatly reduces free heavy metal ion concentrations in the cytoplasm (Rauser, 1995).

Under heavy metal toxicity, the synthesis of another group of Cys-rich peptides has been reported. These peptides are analogous to the gene-encoded metallothioneins found in animals, fungi and cyanobacteria, which have a role in complexing and detoxifying different kinds of heavy metals. In plants, metallothionein synthesis has been reported to be induced by several heavy metals, including Cu in *Arabidopsis thaliana* (Murphy *et al.*, 1997), Zn in wheat (Lane *et al.*, 1987) and Cd in soybean (Blakely *et al.*, 1986).

The synthesis of potential chelating agents in the cytoplasm may raise the question of competition between metals for organic ligands, especially under conditions of hyperaccumulation. Although the basic factors determining the

formation of complexes are thermodynamic stability and concentrations, the prediction of the true complexes occurring at cytoplasmic pH should also consider other aspects. Von Wirén *et al.* (1999) investigated experimentally the formation of NA complexes with Fe^{2+} and Fe^{3+}, and found that although the formation of Fe(III)-NA is thermodynamically favoured, under aerobic conditions the Fe(II)-NA complex is more stable kinetically. Kinetic considerations are also of outmost importance in the formation of other chelate complexes (e.g. with DMA) with different metals. Due to slow kinetics, the formation of Cr(III)-DMA complex is restricted. This demonstrates that metal complexes may preferentially form if favoured by fast kinetics, even if their formation constants are less favorable (Hider 1984).

Concerning the effect of heavy metals on Fe homeostasis, the observations presented above imply that complex formation may be shifted from one ligand to another. For example, it is known that there are special proteins binding and escorting Cu to the appropriate targets. These copper chaperones may function under conditions of normal Cu supply (Field *et al.*, 2002). However at elevated concentrations, as more and more Cu ions are available in the cytoplasm, they may form complexes with ligands such as amino acids and NA (Herbik *et al.*, 1996). As PC synthesis is switched on, Cu would also bind to PCs. In yeast exposed to Ni, Ni(II)-NA complexes have been determined (Vacchina *et al.*, 2003). In Fe deficient sugar beet plants, the level of glutathione, a precursor of PCs, increased 1.6-fold (Zaharieva and Abadía, 2003), whereas under Cd toxicity, which is thought to induce Fe deficiency, PC synthesis is increased (Sanitá di Toppi and Gabrielli, 1999). The transient competition between heavy metals and Fe for chelating agents may substantially modify Fe homeostasis. Complex formation, even if kinetically favoured, is influenced by equilibrium processes, in which thermodynamic stability is the prevalent factor. Also, physiological uptake, transport, assimilation and sequestration processes continuously alter metal and ligand concentrations in the cytosol. For these reasons, direct analysis of metal-chelates existing in plant materials should be strongly encouraged, instead of relying only on theoretical chemical speciation (Hider *et al.*, 2004).

The appearance of non-essential heavy metals in the shoot gave rise to the hypothesis of apoplastic transport (Bell *et al.*, 1991a; White 2001). This pathway has been suggested as a possible rout for Zn hyperaccumulation in *Thlaspi caerulescens* (White *et al.*, 2002). However, in more recent studies, circumstancial and experimental evidence have been presented against a substantial role for apoplastic metal loading (Ernst *et al.*, 2002; Kerkeb and Krämer, 2003). Thus, the translocation of heavy metals as well as Fe likely starts with xylem loading from the symplasm. The molecular background of

xylem loading of heavy metals and Fe is still poorly known. There is, however, substantial analytical data on the concentrations and forms of metals present in the xylem sap. It is well established that Fe is translocated as Fe^{3+} complexed by citrate (Tiffin, 1966). Citrate was also found to be the principal compound chelating heavy metals in the xylem sap of different plant species exposed to Pb or Cd (Senden *et al.*, 1995; Tatár *et al.*, 1998). Concerning the transport of Fe into the xylem, a shift between chelating ligands may occur, since at pH 5.5-6 Fe can be readily released by NA to favour the formation of citrate complexes such as those predicted by chemical speciation studies $[FeCitrateOH]^{-1}$ and $[FeCitrate_2]^{-3}$ (López-Millán *et al.*, 2000). However, since the prevalent Fe form in the cytoplasm could be Fe(II)-NA as indicated previously, xylem loading should theoretically involve an oxidation step to decrease the stability of the complex. Nevertheless, NA was also shown to be present in the xylem sap and was suggested to have a role in Cu transport (Pich *et al.*, 1994; Liao *et al.*, 2000). Obviously, heavy metals may also interact with Fe during transport, modifying their uptake, accumulation and physiological effect in the leaf, especially when they are present at elevated concentrations, such as in cases of hyperaccumulation.

As chelate-assisted phytoextraction requires the application of chelating agents at high concentrations, the transported metal form may change as more and more chelating agents enter the plant. Since membrane integrity may be diminished, at least partially, apoplastic xylem loading may be significant in these cases. Direct measurement of Pb-EDTA in xylem exudates of *Brassica juncea* confirmed that the majority of Pb was transported as Pb-EDTA (Vassil *et al.*, 1998; Epstein *et al.*, 1999).

4. CONCLUDING REMARKS

The interaction of heavy metals with Fe has been studied for a long time, and numerous papers have reported inhibition or stimulation of Fe uptake or disturbances in Fe-dependent physiological processes. The identification of Fe-regulated transporters in recent years has solved decade-long debates, but has also revealed new dimensions in Fe nutrition and regulation research. Crosslinking of heavy metal transport with Fe transport through a series of transporter families, however, makes it more difficult to understand the physiological background of the interactions. Non-essential metals may use transporters of essential ones to enter the cytoplasm due to a broad substrate range. Chelation outside, as well as inside the cell, seems to be a major factor determining metal availability, mobility and toxicity. Switching between chelated and ionic forms or between chelates during uptake and

distribution within the cell is still poorly understood, although much research has been done in this subject. Regulatory processes leading to the homeostasis of Fe and essential metals, as well as to tolerance mechanisms enabling accumulation or hyperaccumulation of toxic ones, is another area requiring more research efforts. There are differences not only between metal hyperaccumulator and non-accumulator species, but also between ecotypes of hyperaccumulator species. More background information will be needed to understand the dynamics of heavy metal transport, in order to help biotechnology to breed new genotypes suitable for phytoremediation in different environmental conditions.

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Chapter 7

PLANT-SOIL RELATIONSHIP: ROLE OF HUMIC SUBSTANCES IN IRON NUTRITION

ZENO VARANINI and ROBERTO PINTON
Dipartimento di Scienze Agrarie e Ambientali, University of Udine, Italy. E-mail: pinton@uniud.it

Abstract: Soil humic substances have been widely regarded as playing a beneficial role in Fe nutrition of plants. This effect has been mainly attributed to the complexing properties of humic molecules which can modify the solubility of Fe by: a) preventing precipitation and subsequent crystallization as well-ordered Fe-(hydro)oxides; b) forming soluble complexes with micronutrients which can then move towards the roots. These Fe sources can be used by both Strategy I and Strategy II plants: while in the first case a role as natural substrate for the inducible Fe^{3+}-chelate reductase can be envisaged, in the latter case a ligand exchange mechanism with plant-borne phytosiderophores is likely to operate. Besides this action, soluble low-molecular weight humic fractions have been shown to positively affect mechanisms involved in plant mineral nutrition, such as the PM H^+-ATPase. Furthermore, it has been frequently observed that contact of roots with humic substances induces an enhancement of root growth and the proliferation of root hairs. This kind of biochemical and morphological changes can effectively contribute to the overall process of Fe acquisition by plants.

Key words: growth stimulation; humic substances; iron availability; plasma membrane; rhizosphere

1. INTRODUCTION

Iron concentration in soil solution of most well aerated soils, as estimated from equilibrium reactions, is generally far below that needed for normal plant growth (Kochian, 1991; Römheld and Marschner, 1986a). However, direct measurements show usually higher levels of Fe in soil solution, due to

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the complexation with soluble organic ligands (Lindsay, 1991). These molecules include organic acids and phytosiderophores (Römheld and Marschner, 1986b) released by the roots, microbial siderophores (Shenker *et al*., 1992) as well as fractions of the humified organic matter (Stevenson, 1991). Humic substances, which account for approximately 60% of soil organic matter, are thought to be present in the soil as molecules of very different molecular mass and degrees of solubility. These molecules can influence plant growth and nutrition at various levels, by affecting physical properties of soil, cation exchange capacity and also behaving as a source of (macro)nutrients (Chen and Aviad, 1990; Varanini and Pinton, 1995). The importance of humic substances lies also in their ability to complex metals in soils, especially cationic micronutrients, thus modifying their solubility. This is especially true for those metals, such as Fe, whose solubility is regulated by the formation of insoluble hydroxides produced through a progressive polynucleation of hydrated ions as the pH increases (Baes and Mesmer, 1976). With regard to Fe availability, the molecular size and solubility of humic substances are important factors to consider. Fractions of higher molecular mass, which are mostly insoluble, can withhold large amounts of the metal, especially in alkaline environments. Iron is thus subtracted from precipitation and subsequent crystallization processes that would decrease its availability (Schwertmann, 1966), and a Fe pool is maintained in equilibrium with complexing molecules present in the soil solution. In addition, soluble humified organic molecules, which are also present in the soil solution (Chen, 1996), can help to increase metal transport by diffusion to the roots (Pandeya *et al*., 1998) and act as natural substrates for root Fe acquisition mechanisms (Pinton *et al*., 1999a). Besides these effects, several studies indicate that humic substances can influence plant metabolism by interacting with a variety of biochemical mechanisms and physiological processes, stimulating growth and increasing the total amount of nutrients taken up by the plant (Vaughan and Malcolm, 1985).

Table 7-1. Elemental contents of humic and fulvic acids (modified from Stevenson, 1994).

	Dry and de-ashed (%)	
Element	Humic acids	Fulvic acids
C	50-60	40-50
O	30-35	44-50
H	4-6	4-6
N	2-6	<1-3
S	0-2	0-2
P	<1	<1

2. DEFINITION, CHEMICAL AND STRUCTURAL CHARACTERISTICS OF HUMIC SUBSTANCES

Humic substances are the result of biological and chemical transformations of plant, animal and microbial residues carried out by soil microorganisms. The resulting chemical compounds are more stable than the precursors. In the soil, humic substances are linked to mineral components through various chemical-physical interactions (Theng, 1979) and mixed with other compounds of different nature. Humic fractions have been found in the soil solution, with a concentration highly variable depending on soil type and ranging from 1 up to 400 mg/L (Chen and Schnitzer, 1978; Cesco, 1995; Gerke, 1997). In order to define the chemical characteristics of humic substances and evaluate their role in plant-soil interactions, several methods have been developed. One of the most commonly used consists of an extraction using strong alkaline solutions (e.g. NaOH 0.1-0.5 N). Although this method is frequently employed because of the high yield of humic substances extracted, its use is questionable, since humic molecules are probably altered during the process, giving origin to possible artefacts (Stevenson, 1994). Several other milder and more selective extractants have been proposed (Stevenson, 1994; Swift, 1996), which are believed to modify the native humic molecules during isolation to a lesser extent. Methods using water to extract soluble humic substances from humus-rich substrates such as peat have also been employed (Pinton *et al*., 1998). Such procedures rely on adsorption onto resins which selectively retain aromatic (phenolic) compounds (Aiken *et al*., 1979) to concentrate and purify the humic molecules.

Table 7-2. Range of distribution of oxygen-containing functional groups in humic and fulvic acids isolated from soils of widely different climatic zones (in mEq/100 g) (modified from Schnitzer, 1977).

	Humic acids	Fulvic acids
Total acidity	560-890	640-1420
COOH	150-570	520-1120
Acidic OH	210-570	30-570
Weakly acidic + alcoholic OH	20-490	260-950
Quinone and ketonic C=O	10-560	120-420
OCH_3	30-80	30-120

Humic substances can be further fractionated into components having different chemical and physical properties. The most common and accepted technique is based on differential solubility in water at various pH values (Aiken *et al*., 1985):

- Humic acid is the fraction that is not soluble in water acidic conditions (pH < 2), but soluble at higher pH values;

- Fulvic acid is the fraction that is soluble in water under all pH conditions;
- Humins is the humic fraction that is not soluble in water at any pH value.

The humic and fulvic fractions can be considered "pure" (although not in a chemical sense) once the salts and, in the case of fulvic acid, the known organic compounds (simple sugars, aminoacids, etc.) that are not structurally bound have been removed.

Certain distinctive features of humic and fulvic acids have been defined, including the elemental composition (Table 7-1), acidity and the presence of oxygen-containing functional groups (Table 7-2).

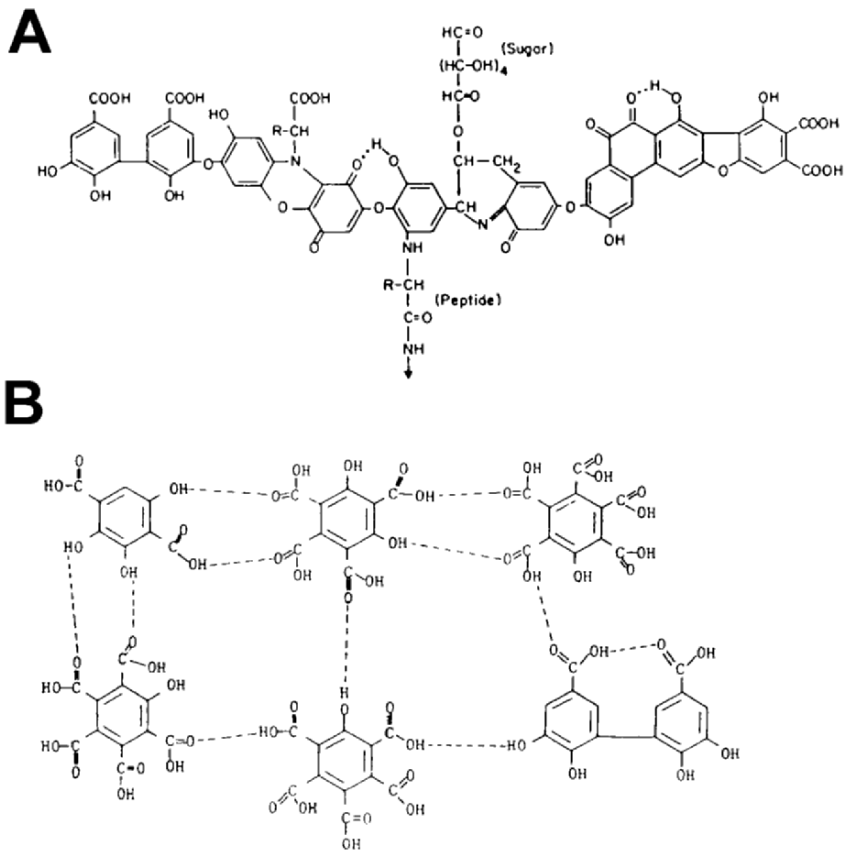

Figure 7-1. Structures of (A) humic acid as proposed by Stevenson (1994) and (B) fulvic acid as proposed by Schnitzer and Khan (1972).

Several analytical and computational methods have been employed to gain precise and satisfactory structural definition of humic substances (Schulten and Schnitzer, 1997). Although none of these approaches has

provided a definitive molecular structure, some information on the structural features of humic substances is available. These molecules contain considerable amounts of single-ring aromatic structures that can interact with each other and with aliphatic chains, therefore forming the skeleton of humic substances (Figure 7-1). The aggregation of these building blocks causes the formation of complexes with molecular dimensions that can vary from a few hundred or a few thousand Da (fulvic acids; Linehan, 1977) up to hundred of thousands (humic acids; Swift and Posner, 1971). More recently, humic substances have been described as supramolecular associations rather than macromolecular polymers; this model implies that small and heterogeneous humic molecules self-assemble in a supramolecular conformation, stabilised mainly by weak forces such as dispersive hydrophobic interactions and hydrogen bonds (Piccolo, 2002).

3. EFFECTS OF HUMIC SUBSTANCES ON IRON CHEMISTRY AND AVAILABILITY

3.1 Complexing properties and reducing capacity

The ability of humic substances to form complexes with cationic micronutrients depends on their content of electron donor functional groups (Stevenson, 1994). Either weak bonds such as water bridges, electrostatic attraction due to cation exchange capacity, or strong bonds involving coordination with single groups or formation of ring structures (chelates) with carboxyl, alcohol, and amino groups can thereby be formed. The formation of different types of bonds depends on the latters' degree of saturation; weaker bonds play a more important role when stronger ones become saturated. The formation of more than one bond between the metal and the organic molecule usually results in a higher stability of the complex. The stability of the metal chelate complex depends on the number of atoms that form a bond with the metal ion, the number of rings that are formed, the nature and concentration of the metal ion, and pH (Stevenson, 1994). The stability order of the complexes formed between metals and humic acids has been determined through potentiometric titration and follows the Irwing-Williams series:

$$Pb^{2+} > Cu^{2+} > Ni^{2+} > Co^{2+} > Zn^{2+} > Cd^{2+} > Fe^{2+} > Mn^{2+} > Mg^{2+}$$

On the other hand, at a pH value of 5.0 there were no large differences in the strength of bonds between humic acids and metals such as Ca, Mg, Mn, Co, Ni and Zn, whereas Pb, Cu and Fe were more strongly bound (Schnitzer and Kahn, 1972); this behavior indicates that at different pH values, metal-humic substances complexes of different stability are formed in the soil. Due

to the heterogeneity of humic substances concerning molecular weight, content of functional groups, variety of bonding sites and changes in conformation of these macromolecules with pH and salt concentration, it is difficult to reach an unequivocal state of knowledge (Chen and Stevenson, 1986). Attempts have been made to determine the apparent stability constants (K_{app}) for Fe^{3+} with two hydrophobic fractions of dissolved organic matter obtained from a manure compost, after sorption onto XAD-8 resin (Chen *et al*., 2004). The K_{app} values at pH 5.0 and an ionic strength of 0.1 M were 7.91 for the fraction desorbed with NaOH and 6.76 for that desorbed with methanol. Garcia-Mina *et al.* (2004) investigated the stability of different metal-humic complexes (NaOH extracted) in the pH range 6-9 and ionic strength of 3 mM, and found maximal K_{app} values (4.11) at pH 8.0. These values are somewhat lower than the maximal stability constant values determined for complexes between Fe and synthetic chelating agents (e.g. EDTA, EDDHA) (Lucena, 2003) or organic compounds of biological origin (e.g. organic acids, siderophores, phytosiderophores) (Von Wirén *et al*., 2000; Crowley, 2001; Ryan *et al*., 2001). However, it should be kept in mind that for compounds like EDTA, citrate and oxalate the ability to maintain Fe in solution above pH 6.0 is very limited. Furthermore, the stability of high levels of organic acids or (phyto)siderophores in soils is unlikely, due to their rapid microbial decomposition. In this context, the contribution of humic substances to Fe availability is conceivably significant, based on the amount of molecules present in the soil and soil solution, the high resistance to microbial degradation, and the relatively high stability constant at pH values typically encountered in alkaline and calcareous soils.

Humic substances are known to be redox reactive and capable of chemically reducing metals including Fe^{3+} (Skogerboe and Wilson, 1981; Struyk and Sposito, 2001). Standard redox potentials for fulvic and humic acids have been evaluated to be around 0.5 and 0.7 V, respectively. It has been shown that reduction of Fe^{3+}occurs at significant levels at pH values lower than 4; at higher pH values reduction is decreased by formation of complexes between Fe^{3+} and humic molecules.

3.2 Iron availability

The Fe pools in soils can generally be divided into: a) ionic and complexed form in solution; b) exchangeable; c) specifically adsorbed; d) organically complexed but water insoluble; e) insoluble inorganic precipitates; and f) held in primary minerals. It is generally acknowledged that organically bound forms are more available to plants than the inorganic insoluble pools. The chemical properties of humic substances, leading to the formation of complexes with Fe and, at least in certain circumstances, to its

reduction, can profoundly affect Fe availability in soils. Complexation of Fe at pH values found in most soils helps maintaining Fe in solution and/or in bioavailable forms. Insoluble humic acids can withhold considerable amounts of microelements including Fe (Linehan, 1985), thereby representing a potential reservoir of such element in soil. Furthermore, in the presence of high quantities of Fe, fulvic acids can form inter-chain bonds producing insoluble macromolecules (Sequi *et al*., 1975).

Humic substances can affect Fe availability also by an indirect mechanism involving the stabilisation of amorphous Fe oxides by high molecular weight humic fractions (Schwertmann, 1991). This process would prevent the re-crystallisation of ferrihydrite to more crystalline oxides, thus keeping Fe in forms more available to plants. The ability of humic substances to complex Fe can also be important for phosphorous nutrition. In fact, phosphate can be bound to humic substances by Fe bridges (Gerke and Hermann, 1992); complexation of Fe by carboxylates such as those released by plant roots (e.g. citrate) can increase both phosphate and Fe solubility (Gerke, 1993). A direct contribution to Fe availability can derive from the formation of water-soluble Fe-humate complexes, which can move in the soil toward the roots (Pandeya *et al*., 1998).

4. ROLE OF HUMIC SUBSTANCES AS NATURAL IRON-CHELATES

Due to their ability to maintain Fe in solution, soluble Fe-humate complexes can act as natural Fe-chelates available for plants. Because of their complexing ability, it is generally accepted that water-soluble humic molecules can mobilise Fe from soil particles to the root surface. The quantitative aspects of this process have not yet been elucidated. It is reasonable to think that the importance of the Fe-humate complexes depends on the humic substances/Fe ratio. As proposed by Linsday and Schwab (1982) for the movement of Fe-EDTA chelate from the solid phase of the soil to the root, the following scenario can be hypothesised in the case of soluble humic molecules: at a low humic substances/Fe ratio, the molecules will tend to mobilise Fe from the solid phases to form stable complexes. If the amount of Fe is not sufficient to form humic macromolecules of lower solubility (see previous section) the soluble complex will move by diffusion toward the roots. Evidence supporting this view has been provided by Garcia-Mina *et al*. (2004) by applying Fe complexed to NaOH-extracted humic acids to three different soils. Cesco *et al*. (2000) observed that a water extractable humic fraction (WEHS), purified from a water extract of sphagnum peat using XAD-8 amberlite resin, can solubilise Fe present as

ferrihydrite and mobilise it in a soil column, making it available for exchange with organic chelating agents such as phytosiderophores released by deficient barley roots. The dynamics of Fe mobilisation by humic substances must, however, take into account the prevailing conditions at the rhizosphere, such as pH and redox potential, and the presence of other type of chelating agents of microbial (siderophores) or plant (organic acids and phytosiderophores) origin. In this context, the different plant strategies in response to limited Fe availability need to be considered. To this respect it is interesting to observe that response mechanisms to Fe-deficiency have been studied almost exclusively using synthetic chelates such as EDTA and EDDHA or, in a few cases, organic acids released by the roots (such as citrate and malate). It is however reasonable to suppose that a mixture of natural chelates is present in the soil and in the rhizosphere (Crowley, 2001). Among natural chelates, humic molecules may play an important role in the mechanisms involved in Fe uptake. Several studies have presented evidence confirming this assumption (Lobartini and Orioli, 1988; Pinton *et al*., 1998; Chen *et al*., 2001). Clear evidence that soluble Fe-humate complexes can be used as a source of Fe has been provided by Pinton *et al.* (1999a). A more rapid or better recovery from symptoms of chlorosis was observed in cucumber plants treated with the Fe-WEHS complex than those treated with other Fe sources (Fe-EDTA, Fe-citrate and $FeCl_3$). Recovery from Fe deficiency after addition of Fe-humate also occurred when plants were grown in nutrient solution at alkaline pH (Figure 7-2) and in the presence of $CaCO_3$ (Mohamed *et al*., 1998). Furthermore, Cesco *et al.* (2002) showed that Fe-sufficient and deficient cucumber plants were able to absorb and translocate to the shoot ^{59}Fe supplied to the nutrient solution as ^{59}Fe-WEHS; uptake and translocation of ^{59}Fe could also be measured at pH 7.5, suggesting that Fe-WEHS could contribute to Fe nutrition even at pH values which are generally associated with low Fe availability in the soil. The Fe^{3+}-WEHS complex (1 μM Fe) could also be reduced by intact roots of cucumber plants either at pH 6.0 or pH 7.5, supporting the view that soluble Fe-humate can act as one of the naturally occurring substrates for the inducible Fe^{3+}-chelate reductase. The Fe^{3+}-WEHS complex (1 μM Fe) was reduced at higher rates as compared to 1 μM Fe^{3+}-EDTA (Pinton *et al.*, 1999a), irrespective of the Fe nutritional status of the plants (Table 7-3). Furthermore, the relative increase in Fe^{3+}-chelate reducing activity induced by Fe deprivation, was considerably different between Fe^{3+}-EDTA (3-fold) and Fe^{3+}-WEHS (8-fold). This result can be particularly interesting in the view of what was observed by Lucena (2003) who showed that the rate of reduction of Fe^{3+}-EDTA by roots of mild-chlorotic cucumber plants was higher than that of other synthetic chelates. However, the higher efficiency of Fe-WEHS as Fe source with respect to synthetic chelates can not be

solely ascribed to a higher Fe^{3+} reduction by roots but also to the capacity of the humic fraction to stimulate proton extrusion (Pinton *et al*., 1999a), a component of the Fe-deficiency response in Strategy I plants.

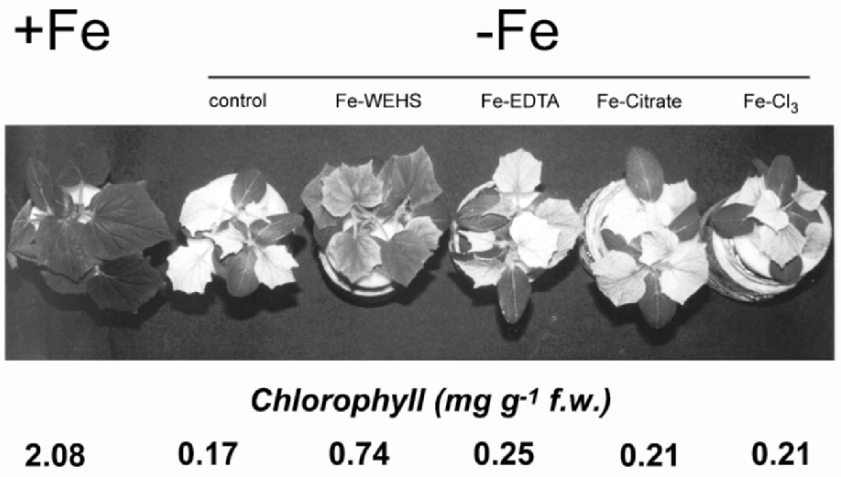

Figure 7-2. Recovery of Fe-deficiency symptoms in cucumber plants after addition of different Fe sources (0.2 µM Fe) to a nutrient solution buffered at pH 7.5 (modified from Pinton *et al.*, 1999a).

^{59}Fe-WEHS could also be used by barley plants (Strategy II) *via* a mechanism which may involve ligand exchange between phytosiderophores and WEHS (Cesco *et al*., 2002). In fact, a high rate of ^{59}Fe-WEHS uptake in Fe-deficient plants could be observed during the period of high phytosiderophore release. The very low rate of ^{59}Fe uptake in conditions of low phytosiderophore release would exclude a massive direct utilisation of the ^{59}Fe-WEHS complex by barley plants. Similar mechanisms have been proposed for Fe uptake from microbial siderophores by graminaceous plants (Yehuda *et al*.,1996).

Based on the above observations, it can be concluded that soluble Fe-humate complexes can be regarded as suitable Fe sources both for Strategy I and Strategy II plants.

Table 7-3. Reduction of Fe^{3+}-WEHS or Fe^{3+}-EDTA (1 µM Fe) by intact roots of Fe-sufficient (+Fe) and Fe-deficient (-Fe) cucumber plants (data from Pinton *et al*., 1999a).

	Growth condition	
	+Fe	-Fe
	nmol Fe^{2+} g^{-1}FW h^{-1}	
Fe^{3+}-EDTA	11.6 ± 0.6 (100)	35.2 ± 1.7 (303)
Fe^{3+}-WEHS	26.1 ± 1.2 (100)	205.4 ± 8.2 (787)

5. ACTION OF HUMIC SUBSTANCES ON MECHANISMS OF I(R)ON UPTAKE AND PLANT GROWTH

5.1 Nutrient uptake

Humic substances have been proven to stimulate plant growth and nutrient accumulation (for review see Vaughan and Malcolm, 1985; Chen and Aviad, 1990; Varanini and Pinton, 1995). Studies on uptake kinetics, use of protein synthesis inhibitors and variations in experimental conditions (e.g. temperature) suggest that the effects of humic substances on plant nutrition may be mediated by variations in the synthesis and functionality of membrane carriers. Moreover, stimulation of active proton extrusion from roots (Pinton *et al*., 1997) and transmembrane potential hyperpolarization (Slesak and Jurek, 1988) indicated an involvement of the plasma membrane H^+-ATPase (PM H^+-ATPase) in the increased nutrient uptake due to the presence of humic substances. Direct proof of an interaction between humic molecules and PM H^+-ATPase has been obtained by Varanini *et al*. (1993) who demonstrated that low molecular weight (< 5 kDa) humic molecules can stimulate the phosphohydrolitic activity of this enzyme in isolated plasma membrane vesicles. Stimulation could also be observed in the presence of the detergent Brij 58, thus suggesting a possible direct interaction involving the scalar activity of the enzyme. On the other hand, experiments on the effects of humic substances on proton transport properties in plasma membrane vesicles showed that membrane permeability to ions was also affected by these molecules, which therefore appear to have a variety of membrane targets. PM H^+-ATPase activity was also shown to be stimulated by a WEHS fraction after short-term (4 h) treatment of maize roots (Pinton *et al*., 1999b); since no increase in protein amount was observed, this effect was attributed to an undefined post-translational mechanism. Canellas *et al*. (2002) showed that a prolonged root treatment (up to 7 d) with humic acids extracted from earthworm compost increased PM H^+-ATPase activities and the protein amount at steady-state. The possibility that humic substances exert an influence at the level of PM H^+-ATPase gene(s) expression was investigated by Quaggiotti *et al.* (2004), who showed an increase in transcript abundance of the PM H^+-ATPase (isoform MHA2) in maize roots treated for 48 h with an earthworm low molecular weight humic fraction. Taken together, these results strongly support the view that humic substances affect PM H^+-ATPase, thus providing an increased proton-motive force for nutrient uptake. This effect can be of relevance for Fe nutrition, since increased PM H^+-ATPase activity can contribute to:

- solubilisation of Fe in the apoplast and in the rhizosphere;
- maintenance of favourable conditions for the activity of Fe^{3+}-chelate reductase (low apoplastic pH and transmembrane electrical potential homeostasis);
- uptake of free Fe^{2+} or Fe^{3+}-complex (e.g. Fe-phytosiderophores).

5.2 Plant growth

Many authors have observed that plants treated with humic molecules have different growth and morphology as compared to control plants (Nardi *et al*., 2002). Treatment with these substances modified root morphology, inducing a proliferation of root hairs in the subapical regions and a higher differentiation rate of root cells. Canellas *et al*. (2002) showed that humic acids extracted from earthworm compost enhanced root growth of maize seedlings and caused a marked proliferation of sites of lateral root emergence. This kind of plant root responses to the treatment with humic substances has been interpreted in terms of a hormone-like activity of humic substances. Recent studies have shown that humic substances extracted from different sources could react to antibodies directed against indolacetic acid (Muscolo *et al*., 1998; Pizzeghello *et al*., 2001). Furthermore, the presence of exchangeable auxin groups in humic substances extracted from earthworm compost was revealed by means of gas chromatography-mass spectrometry (Canellas *et al*., 2002). On the other hand, Chen *et al.* (1994) failed to provide evidence of the presence of phytohormones in humic extracts. In an attempt to clarify whether humic molecules act as auxin-like substances, Schmidt *et al*. (unpublished) analysed the response of an array of *Arabidopsis* auxin-related mutants to the presence of a water extractable humic fraction (WEHS). Root hair density was significantly increased by the presence of WEHS in the wild type. The phenotypes of the mutants, all exhibiting a reduced number of root hairs, were not rescued by the application of WEHS, suggesting that functional products of the auxin signalling cascade are required for translating the response of root cells to the humic molecules. In addition, mutants defective in root hair initiation *rhds6*, known to develop normal hairs in the presence of auxin, were not affected by a wide range of applied concentration of WEHS indicating that this humic fraction cannot substitute for the hormone. These results show that, at least for WEHS-like humic fractions, a modification of root hair density can be induced in a manner different from that exerted by exogenous auxin.

The increased root hair density, similar to that occurring at sub-optimal availability of immobile nutrients such as Fe (Müller and Schmidt, 2004), support the view that WEHS-like humic fractions induce a “nutrient

acquisition response" that favours nutrient capture via an increase in the absorptive surface area.

A schematic representation of the possible interactions between soil humic substances and mechanisms related to Fe acquisition is shown in Figure 7-3.

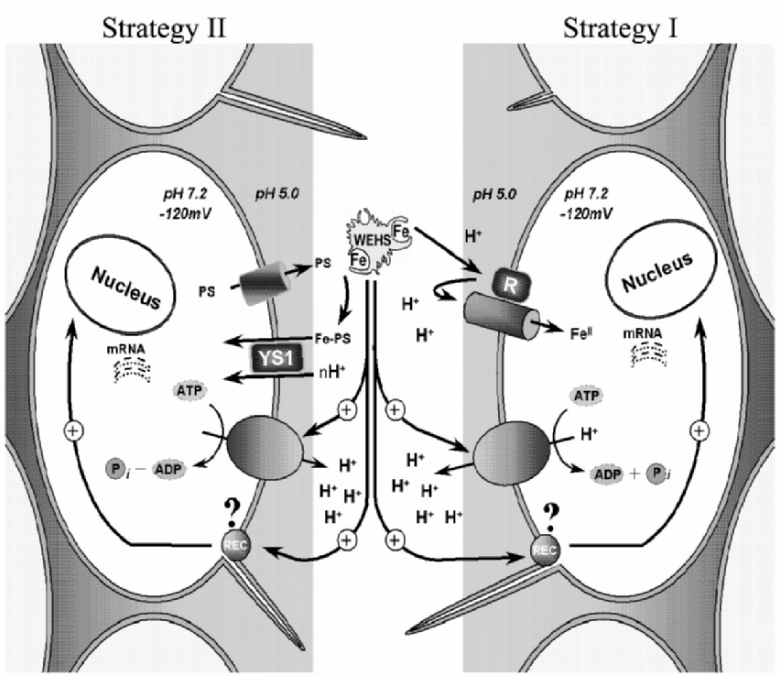

Figure 7-3. Schematic representation of possible interactions among humic substances and mechanisms involved in Fe acquisition in roots of Strategy I and II plants. R: inducible Fe^{3+}-chelate reductase. REC: hypothetical receptor for water-soluble humic molecules.

6. CONCLUSION

Evidence presented supports the view that humic molecules can play both a direct and an indirect role on plant growth and nutrition (Varanini and Pinton, 2001). It has been unequivocally proved that fractions of the humified organic matter can complex Fe, behaving as natural chelates. Further studies are needed to better define their contribution to Fe nutrition in conditions closer to those encountered in the soil and/or at the rhizosphere, where interactions with other natural (plant- or microbial-borne) chelates conceivably occur. Furthermore, the physiological action *per se* of humic molecules on membrane activities relevant for Fe nutrition

makes these substances quite different from the chelates which have been normally used in most Fe nutrition studies, and suggests that their direct contribution should be considered when the plant response to Fe shortage is under evaluation. Further progress may derive from a more accurate definition of the chemical and physical properties of humic substances, including the presence of bioactive compounds and how do they interact with the root cell apoplast and the plasma membrane. An interaction with the PM H^+-ATPase which could lead to an enhanced Fe acquisition has already been observed: regulatory aspects of this interaction need to be further investigated at the molecular level, considering both transcriptional and post-translational regulation. However this 'master enzyme' may not be the sole molecular target of humic compounds: other proteins (enzymes or carriers) could be involved in the regulation of Fe uptake. The use of plant mutants appears to be a promising avenue for clarifying the mechanisms underlying the promoting effect of humic substances on plant growth and Fe uptake.

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Chapter 8

MICROBIAL SIDEROPHORES IN THE PLANT RHIZOSPHERE

DAVID E. CROWLEY
Department of Environmental Sciences, University of California, Riverside, CA 92521, USA.
Email: crowley@ucr.edu

Abstract: Siderophores are iron chelating agents that are produced by almost all microorganisms in response to iron deficiency. Due to the requirement of iron for cell growth and metabolism, siderophore mediated acquisition of iron plays a central role in determining the ability of different microorganisms to colonize plant roots and contributes to microbial interactions in the plant rhizosphere. There are now approximately 500 known siderophores, some of which are widely used by a variety of microorganisms, whereas others are used only by the same microbial species and strains that produce them. Siderophores also have been examined for their potential importance in plant iron nutrition and for their ability to mobilize heavy metals. There has been considerable debate over their relative importance for plant iron nutrition that has centered around their ability to release iron by means of chelate reductases that function in Strategy I plants and to exchange iron with phytosiderophores in Strategy II grasses. A key question concerning the relevance of siderophores in plant nutrition has been whether siderophores are produced in sufficient quantities by microorganisms to supply physiologically relevant quantities of iron to plants. Siderophores are generally detected in low concentrations in soil extracts. Molecular methods using reporter genes further show that siderophore production is limited to sites of high microbial activity. There is now general recognition that siderophores have a fundamental role in determining the bioavailability of iron to microorganisms that colonize the rhizosphere and are required for rhizosphere competence in microorganisms that are being developed for biocontrol of plant diseases and plant growth promotion.

Key words: chelate; disease suppression; iron; microbial ecology; plant growth promoting bacteria

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1. INTRODUCTION

Iron is one of the most commonly limiting trace elements in nature but is essential for the growth and metabolism of almost all living organisms. Deficiencies of iron are primarily due to the poor solubility of this trace element with increasing pH such that above pH 4, almost all microorganisms have evolved highly specific systems that employ iron chelating substances, called siderophores, that are secreted into the environment to dissolve iron minerals and hold it in a soluble form that can move by diffusion and deliver iron to the cell surface. Due to the wide array of mechanisms and iron chelating agents that are used by microorganisms for acquiring iron, competition for this element can occur between different species of microorganisms that coexist in the rhizosphere. This has important ramifications in the rhizosphere where the ability to acquire iron can influence both the species composition of the rhizosphere microbial community and availability of trace metals to plant roots. There is now a large body of primary literature on the role of siderophores in various aspects of soil chemistry, microbiology and plant nutrition. Earlier reviews have integrated this knowledge into conceptual models of how siderophores function in the plant rhizosphere (Crowley *et al.*, 1991; Buyer *et al.*, 1994). These models are now being born out by studies exploring microsite phenomena using molecular methods with reporter genes to examine microbial iron stress responses. Siderophores appear to be particularly important for contributing to rhizosphere competence of plant growth promoting and disease suppressive bacteria. These compounds are also now being examined in relation to other aspects of the rhizosphere including heavy metal uptake and bioremediation of soil pollutants. This chapter provides an overview of the general role of microbial siderophores in rhizosphere microbial ecology and recent studies that contribute to our understanding of the role of siderophores in the rhizosphere.

2. MOBILIZATION OF IRON BY SIDEROPHORES

2.1 Iron availability in the plant rhizosphere

Iron availability in the plant rhizosphere is limited by the extreme insolubility of inorganic iron minerals that dissolve at much slower rates than are required to support plant and microbial growth (Lindsay, 1995). The solubility of inorganic iron is controlled both by the pH and redox of the soil solution and involves a series of iron hydrolysis species that are in equilibrium with iron bearing minerals that have different solubilities that

reflect their crystallinity and stability, the most soluble being amorphous iron hydroxide and the least soluble being goethite. The amounts of soluble iron that are maintained by equilibrium with iron minerals have been modelled and can be predicted for specific pH and redox conditions (Figure 8-1).

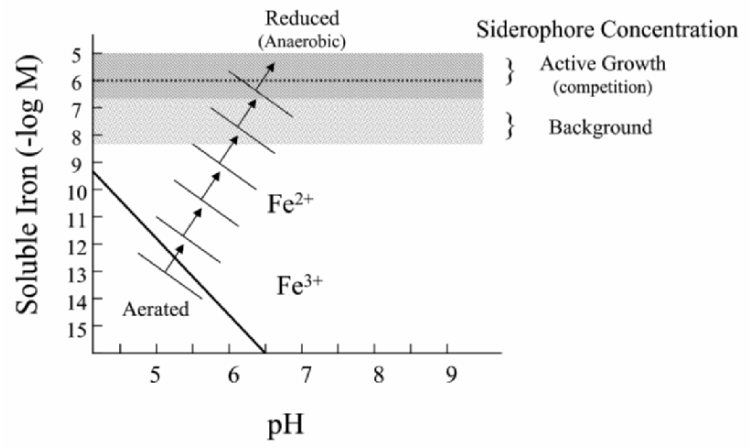

Figure 8-1. Solubility of inorganic iron in soils as controlled by pH and redox. Diagram is simplified only to show Fe^{3+} ion (solid line) and changes in Fe^{2+} (short dashed line), the latter which becomes increasingly soluble under reduced conditions (Solubility diagram adapted from Lindsay and Schwab, 1982; Crowley, 2001).

As shown in Figure 8-1, the soluble concentrations of the two main hydrolysis species, Fe^{3+} or Fe^{2+}, are well below the levels that are required by plants and microorganisms. In addition to these two hydrolysis species, there are several others in which the dissolved iron is coordinated by water molecules that dissociate to different degrees to generate $Fe(OH)_2^+$, $FeOH^{2+}$, and $Fe(OH)_3^o$ (Lindsay, 1979). In the soil solution, the $Fe(OH)_2^+$, $FeOH^{2+}$, Fe^{3+}, hydrolysis species of iron all have a similar solubility of approximately 10^{-6} M at pH 3 and decline thereafter by factors of 10, 100, and 1000-fold, respectively, for every unit increase in pH. Above pH 7, under aerated conditions, the only iron hydrolysis species with any relevance for diffusion of inorganic iron in the soil solution is the neutral hydrolysis species $Fe(OH)_3^o$ which does not change with respect to pH, and which maintains a constant equilibrium concentration of approximately 10^{-10} M across all pH values. In contrast to the low concentrations of soluble iron maintained by equilibrium with mineral iron, plants and microorganisms require approximately 1-10 µM soluble iron to meet the normal demand for this element during active growth. This is approximately 10,000-fold more than

the concentration of inorganic iron in solution that is maintained above pH 4 under aerated conditions.

To solve this problem, plants and microorganisms respond by several different mechanisms to increase the solubility of iron. Strategy I dicots employ reductases and excrete protons to acidify the rhizosphere and lower the redox conditions to increase the solubility of inorganic iron. Microorganisms and Strategy II monocots, on the other hand, rely on organic molecules to increase the solubility of iron. These molecules, called siderophores, are released into the environment to scavenge the low levels of iron in solution and also attack the surfaces of iron minerals to directly mobilize iron from the solid phase minerals. Siderophores may also remove iron from organic iron complexes and stable humic and fulvic acid organic matter where iron is held in complexes with various ligands. Iron availability *via* siderophores thus involves interactions of these molecules with all of the various sources of iron that are present in the environment as well as between the chelators themselves which may strip iron from other siderophores by ligand exchange. With the exception of the soluble iron in solution, which is chelated almost instantly by these compounds, all other processes involve ligand exchange or dissolution reactions that are much slower and dependent on kinetic processes that are not easy to predict and must be measured empirically. This makes modelling of siderophore function in the environment very difficult to predict, especially in complex mixtures where many factors may influence kinetic processes involving iron dissolution and ligand exchange.

As shown in the conceptual model in Figure 8-2, several pools of iron can potentially be accessed by siderophores. The primary source for replenishing these pools of iron is the solid phase minerals, which represent the largest reservoir of iron in the soil. The dissolution of iron from solid phase iron minerals will have different kinetic rates depending on the crystallinity and surface area of the predominant iron minerals. In addition to the solid phase iron minerals, another source of relatively labile inorganic iron is adsorbed to exchange sites on partially decomposed plant detritus and on stable organic matter in the form of humin, humic and fulvic acids, or on the glycoprotein glomalin, which is produced by mycorrhizal fungi. In the stable organic matter fraction, both humin and humic acids are insoluble, but provide cation binding sites where iron may be tightly complexed but still available for complexation with organic acids or siderophores depending on how well the iron is protected within the organic complexes. Large molecular weight organic complexes may shield access by siderophores, whereas smaller fulvic acid complexes with iron may be more accessible.

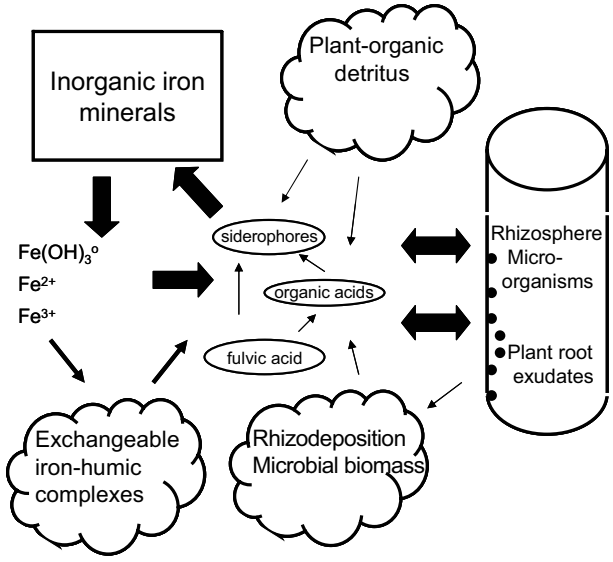

Figure 8-2. Conceptual model of iron mobilization by siderophores showing the different sources of iron that are potentially mobilized including inorganic iron, iron in plant detritus and microbial biomass, and in iron humic complexes with organic matter. Iron may also be exchanged from weak complexes with fulvic acid and organic acids in the soil solution or that are adsorbed in clay organic matter complexes.

Iron also is associated with various organic molecules such as heme and in iron sulfur proteins in the lysates that are generated from turnover of the microbial biomass and in plant detritus. Lastly, some portion of the potential iron chelating substances including siderophores, themselves, and organic acids will be adsorbed to clay and organic matter complexes through hydrophobic and ionic interactions, and thus may represent a relatively immobile phase of potentially available iron that may be remobilized by exchange with more polar or neutral siderophores and iron chelating substances.

The mechanisms by which siderophores mobilize iron from iron bearing minerals have been recently reviewed by Kraemer (2004), which provides a detailed view of the surface chemistry of iron oxides and iron complex formation by siderophores. At a molecular level, the actual means by which iron is liberated involves a stepwise process in which iron atoms are stripped from the exposed surfaces of iron minerals. Inorganic iron is found in a variety of solid phase minerals including clays, iron phosphates, and iron oxides that vary in their crystallinity and solubility. The iron oxide minerals occlude large amounts of iron within the mineral itself, but on the surface contain iron atoms that are arranged in an octahedral coordination with other

atoms in the crystal lattice and with hydroxyl groups and water molecules that are strongly adsorbed in a monolayer on the mineral surface. Siderophores and certain organic acids that complex iron enter the water layer and form inner sphere complexes with iron atoms by attaching one or more ligands to the mineral surface that donate a shared electron to the iron atom and draw it into a new set of ligands that are provided by the siderophore. Once iron is completely removed from the mineral in a new octahedral complex, the siderophore is free to diffuse away and transport iron along a diffusion gradient to the surface of cells that remove iron from solution by uptake across the cell membrane, or that cleave iron from the siderophore by reducing Fe^{3+} to Fe^{2+} which is not held as tightly by the siderophore. The ability of the siderophore to diffuse without becoming sorbed onto other surfaces depends on its charge and hydrophobicity, which determine the partitioning of the iron chelate between the soil solution and adsorption onto clay and organic matter.

The rates of iron oxide dissolution vary with respect to their crystallinity, ranging from amorphous iron oxides that are relatively easy to dissolve to goethite and hematite, which have very slow dissolution rates. Iron oxides and clay silicates are often associated with humic and fulvic acids, organic acids, and polysaccharides in clay-organic matter complexes that can further affect their dissolution rates and surface chemistry. To date, there are only a few model systems that have been studied, in which the kinetics of iron dissolution from different solid phases has been determined empirically for a few representative siderophore and phytosiderophores types and with a few representative synthetic or natural iron oxides. As compared to chelation with soluble iron which is instantaneous, the surface dissolution kinetics of iron oxides is relatively slow and may take hours to days for all of the siderophore to become complexed with iron. The rates of dissolution will depend on the pH and ionic composition of the solution and relative amounts of siderophore in comparison to the surface area of iron oxides. Overall, there is a linear relationship between the surface excess of siderophores and the dissolution rates of iron oxides, such that dissolution rates increase proportionately with the concentration of siderophore (Cheah *et al.*, 2003). In other words, at low concentrations the dissolution rates will be lower than when there is a large concentration of deferrated siderophore. The deferrated siderophore that accumulates in solution suppresses the concentrations of iron hydrolysis species in the solution to extremely low concentrations such that the only available iron is that which is chelated with siderophores. This establishes competition for iron depending on the ability of an organism to use the predominant siderophore types.

The second important source of iron that is potentially mobilizable by siderophores is that which is held by organic matter complexes either in

stable organic matter fractions including humic and fulvic acids or in plant detritus. Siderophores are capable of mobilizing iron that is contained in humic acids, and have much greater specificity and stability with iron than soil organic matter. Access to this iron, however, can be limited by the structural complexity of soil organic matter where iron may be occluded by the surrounding matrix. Humic acids containing high amounts of aromatic constituents are apparently less accessible than those containing more aliphatic hydrocarbons, which reflect differences in the structural complexity of humic substances (Piccolo *et al.*, 1993).

Along with humic substances, plant detritus is also an important iron source, some of which may be mobilized by siderophores and recycled into the biomass before precipitation as inorganic iron minerals (Mazoy and Lemos, 1991; Chen *et al.*, 2000). Plants contain iron in many different redox proteins that have metal centers involving heme or iron sulfur complexes. This iron can be accessed during degradation of proteins, which release the soluble heme iron complex into the soil solution. Some microorganisms apparently have the ability to transport heme-iron which would negate the need for production of siderophores (Nienaber *et al.*, 2001). Conversely, other compounds that are produced during plant decomposition such as polyphenols hold iron in a form that is accessible only to siderophore producing microorganisms (Mila *et al.*, 1996). In this manner, soils that are rich in organic matter, or that have been amended with iron supplemented organic matter amendments provide an important source of iron that can be mobilized for uptake by microorganisms and possibly by plants that use siderophores directly or indirectly for iron nutrition. The impact of organic matter on reducing competition for iron between microorganisms and in reducing the amounts of siderophore that are needed to obtain iron also has been examined for composts (De Brito Alvarez *et al.*, 1995). Higher numbers of siderophore producers were found in composts that were suppressive to an array of pathogenic fungi, which suggests that siderophores contained in composts could have a role in antagonism of plant pathogens by iron deprivation or are necessary to survival and efficacy of bacteria that are antagonistic to plant pathogens. There is clearly a need for more research on this topic to improve compost use for biocontrol of plant diseases.

2.2 Siderophore producing microorganisms

Almost all microorganisms that have been cultured have been found to produce siderophores, and there are now approximately 500 known siderophore structures (Boukhalfa and Crumbliss, 2002). Although a large number, it is likely an underestimate of the true diversity of siderophores

structures as the vast majority of soil microbial species have not been cultured. Extensive lists of the different siderophores that have been purified and chemically characterized have been provided in prior reviews. Based on their structural features and types of ligands, there are four main classes of bacterial siderophores and three classes of fungal siderophores (Höfte, 1993). The main chemical classes of microbial siderophores are summarized in Table 8-1.

Table 8-1. Siderophore classes and representative siderophore types produced by soil microorganisms (adapted from Höfte, 1993).

Bacterial siderophores			
Phenol catecholates		enterobactins	*Escherichia coli*
			Enterobacteriaceae
		linear catechols	*Agrobacterium*
		mycobactins	*Mycobacteria* spp.
		pyochelin	*Pseudomonas aeruginosa*
		tropolone	*P. plantarii*
Hydroxamates			
	Citrate type	schizokinen	*Bacillus megaterium*
		aerobactin	*Aerobacter*
			Enterobacteriaceae
		arthrobactin	*Arthrobacter* spp.
	Ferrioxamines	ferrioxamine	*Actinomyces* spp.
			Streptomyces spp.
			Nocardia spp.
			Arthrobacter spp.
	Cyclic Di-	alcaligin	*Alcaligenes* sp.
	Cyclic Mono	cepabactin	*P. cepacia*
Carboxylate		rhizobactin	*Rhizobium meliloti*
Pyoverdines		pyoverdine	*Pseudomonas* sp.
		Pseudobactin	*Pseudomonas* sp. St B10
Fungal siderophores			
Rhodotorulic acid		rhodotorulic acid	*Rhodotorula*
			Cryptococcus
			Ustilago
		dimerum acid	*Verticillium* sp.
			Fusarium sp.
			Gaeumannomyces
		coprogen	*Penicillium*
			Neurospora
Ferrichromes		ferrichrome	*Aspergillus*
			Penicillium
Fusarinines		fusarinine	*Fusarium*
			Aspergillus
			Penicillium

The first class of bacterial siderophores described in Table 8-1 are the phenol-catecholates, including enterobactin, which has the highest known

affinity for iron, but which is extremely labile and subject to rapid hydrolysis. Enterobactin is produced by *E. coli* as well as by *Enterobacter* that commonly associate with plants including the plant pathogen *Erwinia carotovora*. Related compounds include the linear catechol agrobactin, which is produced by *Agrobacterium tumefaciens,* and dihydroxybenzoic acid derivatives that are produced by *Erwinia*, *Bacillus subtilis,* and *Pseudomonas stutzeri.* Phenol catecholates in this class also include mycobactins that are produced by *Mycobacterium*, *Nocardia*, and *Rhodococcus* (Ratledge, 1987), and pyochelins that are produced by various species of *Pseudomonas*. Mycobactins, the remaining subgroup in this class, are derived from salicylic acid and are produced by both saprophytic and pathogenic species of mycobacteria. The second major class of bacterial siderophores is comprised of the hydroxamates, which include several subgroups represented by schizokinen, aerobactin, and ferrioxamines. These siderophores are produced by *Enterobacteriaceae*, actinomycetes, and *Arthrobacter* spp., all of which are common colonizers of the plant rhizosphere. The third class is represented by rhizobactin, which is produced by *Rhizobium* sp., and is known to be a highly effective iron source for plants. The last class of bacterial siderophores is comprised of the pyoverdine siderophores, which are mixed ligand siderophores. The structure of these siderophores consists of a peptide chain having three types of ligands: a hydroxamate group, an α-hydroxy acid, and a catechol. These chelators are produced primarily by pseudomonads and *Azotobacter*, and are especially important for increasing the rhizosphere competence of pseudomonads that suppress phytopathogenic fungi. Pyoverdines have also been investigated for their role in plant nutrition, but do not appear to have a major role as potential iron sources for plants.

Major classes of fungal siderophores include three types, the rhodotorulic acids which are di- or tri-hydroxamates, the ferrichrome type siderophores, and the fusarinines. Although produced by fungi, many of the hydroxamate siderophores are also utilized by bacteria and can serve as potential iron sources for plants. Use of these compounds for plant iron uptake can involve either a specific iron uptake system, or a ferrochelate reductase that reduces ferric iron to the less tightly held ferrous ion, which is then transported across the cell membrane (Lodge, 1993). Common soil fungi that produce hydroxamate siderophores include *Penicillium* and *Aspergillus*, which are some of the most commonly isolated fungi in soils. Hydroxamate siderophores are also produced by ectomycorrhizal fungi and ericoid fungi. Although the latter are typically associated with plants in acidic soils where iron deficiency would not be expected to occur, ericoid fungi colonize decomposing wood where iron may be unavailable (Haselwandter *et al.*, 1994). It has also been speculated that siderophore production by mycorrhiza

fungi may promote solubilization of iron phosphates, thereby promoting increased phosphorus availability to plants and microorganisms in phosphate limiting environments in moderately acid soils (Jayachandran *et al.*, 1989).

3. SIDEROPHORE PRODUCTION IN THE RHIZOSPHERE

3.1 Measurements of siderophore production in the rhizosphere

Siderophore producing bacteria are commonly isolated from the plant rhizosphere and can be shown to produce iron chelating substances by growth on indicator media such as chrome azurol S (Koedam *et al.*, 1994; Milagres *et al.*, 1999; Calvente *et al.*, 2001). In general, there do not appear to be any differences in the population densities of culturable, siderophore producing bacteria in the rhizosphere of different plant species (Alexander and Zuberer, 1991). The wide ability of microorganisms to produce siderophores suggests that these compounds are secreted into the rhizosphere, where they may accumulate in a milieu of different siderophore types. Major limitations in measuring the accumulation of siderophores in the rhizosphere are the lack of specific detection methods for more than a few different types of siderophores, and the requirement for soil extraction which dilutes siderophores into large volumes and provides an opportunity for loss by adsorption on to soil and organic matter. The most widely used methods employed siderophore auxotrophic bacterial strains that respond to broad classes of siderophores (Szaniszlo *et al.*, 1981; Powell *et al.*, 1983). Other methods include antibodies for specific siderophore types, and the use of reporter genes which can be correlated with iron stress and used to estimate potential siderophore production. Comprehensive methods have been developed to characterize the composition of root exudates and phytosiderophores, which do not require prior knowledge of what compounds are present in order to be detected (Fan *et al.*, 1997). However, these methods have not yet been applied for detection of microbial siderophores in rhizosphere soil solutions.

When iron is not available in sufficient quantities to support growth, the iron stress responses that are induced in both plants and microorganisms are generally proportional to the relative demand for iron. Under extreme iron deficiency in pure culture, rapid growing microorganisms like pseudomonads may allocate most of their carbon and energy to siderophore production and produce large amounts of siderophores that exceed the weight of the cell biomass in overnight cultures. Whether this occurs in the rhizosphere is less

certain since the availability of carbon and degree of iron stress that is experienced by microorganisms is almost certainly much lower than what occurs in pure cell cultures in iron limiting, rich media. The requirement for siderophore production in the rhizosphere will depend to a large extent on the efficacy of the plant iron stress responses which function to increase iron availability to both plants and the rhizosphere microflora (Marschner *et al.*, 1997; Marschner and Crowley, 1998).

One of the major considerations in evaluating the relative importance of plant and microbial iron stress responses for mediating iron availability is the difference in the overall scale at which plants and microorganisms react to iron stress. Microorganisms will have their greatest iron demand during rapid growth on root exudates and lysates that occurs behind the root apices and at sites of lateral root emergence. If iron is limiting in these particular microsites, siderophore production would be induced to the extent that is necessary to obtain adequate iron for growth. Plants on the other hand respond to iron deficiency by inducing iron stress responses that will globally affect the availability or iron to both the plant and rhizosphere microflora. If one portion of the plant root system has access to iron in sufficient quantities to supply the shoot, or receives a foliar application of iron, the plant iron stress response will not be induced and microorganisms are more reliant on their own iron stress response. The plant responses involving acidification in Strategy I plants and phytosiderophore production in Strategy II plants are expressed primarily along a short region of the root axes behind the root tips. This is also the same location where root exudates are most abundant and microbial growth is most active. Thus the plant responses are occurring in the same zones where microorganisms would have the greatest demand for iron and may be able to rely on the plant response.

Microbial demand for iron and plant responses to iron deficiency will also have a seasonal component. The amounts and type of root exudates that are released into the rhizosphere depend on the plant growth stage, availability of water, light, and nutrients, the soil temperature, and soil physical factors such as bulk density and aeration that influence root growth. Other sources of carbon that might cause microbial iron demand and siderophore production include carbon from rhizodeposition of sloughed cortex cells and from death and turnover of fine roots. Predicting the amounts of siderophore production and determining the predominant mechanisms by which plants and microorganisms obtain iron from different sources is thus likely to be highly variable under different environmental conditions or seasonally influenced by changes in carbon inputs into the rhizosphere during plant growth. Most studies on plant use of microbial siderophores have focused specifically on early plant development in studies

using rapid growing annual plants in hydroponic cultures where siderophores are supplied at fixed concentrations as a potential iron source for plants. On the other hand, the importance of siderophore accumulation in rhizosphere of slow growing perennial plants, shrubs, and trees where fine roots persist for up to a year, and regrow year after year in the same macropores is another consideration that has largely been ignored.

Early attempts at estimating siderophore concentrations in the rhizosphere focused on comparison of the siderophore concentrations in water extracts of rhizosphere and bulk soils. Since purification and analytical procedures need to be tailored for different types of siderophores and there is no *a priori* information on what siderophores should be considered, bioassay methods for general classes of siderophores provide the most practical and sensitive approach. The earliest bioassays used the siderophore auxotroph *Arthrobacter* JG-9, which is responsive to the addition of hydroxamate siderophores that are contained in soil extracts (Szaniszlo *et al.*, 1981; Powell *et al.*, 1983; Reid *et al.*, 1984). Using this method, studies were later conducted to examine the ecology of hydroxamate siderophores in different soils and the effects of nutrient amendments on siderophore concentrations (Bossier and Verstraete, 1986). Under nutrient rich conditions the values for hydroxamate siderophores are reported to range from 20 to 200 μg ferrioxamine equivalents per kg of soil. This corresponds to a concentration of 0.03 to 0.3 nmol/kg of soil when averaged over the entire soil mass. However, the actual concentrations at microsites where siderophores are produced may be much greater. Assuming that these siderophores are all dissolved in solution at 10% soil moisture, this would equivalent to 0.3 to 3 μM concentration. Another consideration is the efficiency in extracting siderophores from the soil. In the case of ferrioxamine B, more than 98% of the siderophore is sorbed to clay and organic matter when it is added directly to soil (Reid *et al.*, 1984). When siderophores are mixed with the entire soil mass, there may be sorption and loss of the compounds from the solution phase. Thus the actual quantities contained in soil solution may be underestimated depending on the efficiency of the extraction procedures.

After initial reports indicating low concentrations of siderophores in soils even under conditions with high microbial activity, there was debate over whether the levels that were measured would be important for supply of iron to plants. As a basis of comparison, plants generally require 10 to 50 μM soluble iron when complexed either with siderophores or with synthetic iron chelators, when grown in hydroponics (Crowley *et al.*, 1988). The low concentrations of siderophores that are detected in soils are not at concentrations that would be sufficient to support plant growth. On the other hand, there may be localized sites in the rhizosphere where siderophores occur at high concentrations where they may contribute to plant uptake of

iron. The various assays on plant use of siderophores and concentrations of particular classes of siderophores in soils have mainly focused on only a small number of siderophore types, some of which are not readily used by plants and others that are excellent sources of iron but which have not yet been assayed to determine their concentrations in soil (Table 8-2).

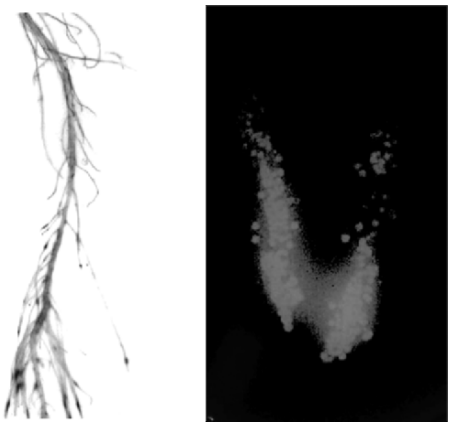

Figure 8-3. (Left) Autoradiograph of oat roots provided with radiolabelled ferrioxamine B showing uptake by microorganisms in the vicinity behind the root tips and at sites of lateral root emergence. (Right) Root fingerprints of fluorescent pseudomonads on agar media showing localization of fluorescent pseudomonads in the area behind the root tip and the capacity of these bacteria to produce large amounts of fluorescent siderophore when provided with abundant carbon.

The localization of siderophore production in the rhizosphere can be ascertained indirectly by use of root fingerprinting techniques in which roots are pressed on to agar media to transfer the adhering bacteria that then grow on the surface of the agar. As shown in Figure 8-3, when roots are pressed on to an agar medium that is selective for fluorescent pseudomonads, most of the colonies that first appear on the agar medium are those that were associated with the zone of elongation behind the root tips. This is the area where root exudates are produced in large quantities and support rapid growth of opportunistic bacterial species such as fluorescent pseudomonads. When the plate is visualized by UV fluorescence, the siderophore production by the bacteria on the agar medium is indicated by light that is given off by the fluorescent siderophore that is secreted by the bacteria. These methods indicate the high potential for siderophore production under nutrient rich conditions and demonstrate the local nature of this phenomenon on root surfaces.

Table 8-2. Experimental studies examining microbial siderophores as iron sources for plants (listed chronologically).

Plant	Siderophore	Conclusions	Reference
Oat	Ferrichrome	Effective for supply of iron to oat at 10 μM concentration	(Crowley *et al.*, 1988)
Pine	Ferrioxamine B	Uptake inhibited in mycorrhizal roots as compared to nonmycorrhizal pine	(Leyval and Reid, 1991)
Oat	Inoculation with pseudomonads	No effect on plant iron uptake	(Alexander and Zuberer, 1993)
Cucumber	Ferrioxamine B	Uptake of Fe-siderophore complex under axenic growth conditions	(Wang *et al.*, 1993)
Barley	Pseudobactin	More effective than EDDHA, but less effective than phytosiderophores	(Duijff *et al.*, 1994)
Carnation	Psuedobactin	Differences in use by plant cultivars related to iron-reduction response	(Duijff *et al.*, 1994)
Cucumber, Maize	Pseudobactin	Poorly utilized as compared to synthetic chelates or phytosiderophores	(Walter *et al.*, 1994)
Grape	Coinoculation of pseudomonads and mycorrhizae	Reduced iron chlorosis	(Bavaresco and Fogher, 1996)
Onion	Ferrioxamine B	Uptake of Fe-siderophore complex, no significant extracellular reduction	(Manthey *et al.*, 1996)
Cucumber Maize	Coprogen, Dimerum acid and Fusarinine	Readily used by both Strategy I and Strategy II plants via reduction and ligand exchange, respectively	(Hoerdt *et al.*, 2000)
Soybean	Aerobactin	Uptake of Fe-aerobactin complex. No external reduction. Uptake inhibited by Na-azide	(Chen *et al.*, 2000)
Tomato, Cucumber, Barley, Corn	Rhizoferrin	Effective iron chelate, used by extracellular reduction of Fe by Strategy I plants and by ligand exchange with phytosiderophores in Strategy II plants	(Yehuda *et al.*, 1996; Yehuda *et al.*, 2000)
Cucumber	Rhodotorulic acid Ferrioxamine B	Fe-rhodotorulic acid slowly used by external reduction mechanism, Fe-FOB not used by this mechanism, nor affected by plant Fe status	(Johnson *et al.*, 2002)

Another method to estimate siderophore concentrations uses antibodies for the siderophore produced by *Pseudomonas* strain B10 (Buyer *et al.*, 1990; Buyer *et al.*, 1993). Pseudobactin is important for the rhizosphere competence of certain plant growth promoting pseudomonads (Duijff *et al.*, 1993; Moenne-Loccoz *et al.*, 1996), and has been implicated in antagonism of plant pathogenic fungi that are deprived of iron when grown in the presence of this siderophore (Duijff *et al.*, 1993; Moenne-Loccoz *et al.*, 1996). Pseudobactin also has been examined as a potential iron source for plants and can support plant iron acquisition when provided at 5 to 10 μM concentration (Duijff *et al.*, 1994a; 1994b). Confirmation of the occurrence of this siderophore in the rhizosphere and determination of its concentration is thus of considerable interest. An advantage of the antibody detection methods as compared to the previously described bioassay methods is that they are highly specific for an individual siderophore type and are extremely sensitive to low concentrations of the target compound.

In experiments using the antibody detection methods, siderophore production was quantified in soil planted with barley and inoculated with 10^9 cfu g^{-1} soil of the bacterium (Buyer *et al.*, 1993). After 3 days, a concentration of 3×10^{-10} mol pseudobactin per gram of rhizosphere soil was found. This is a low concentration that would be of little relevance to either plant or microbial iron nutrition when diluted out over the entire soil volume. There are several additional factors to take into account when determining the relevance of this quantity of siderophore. First, the highly specific nature of the antibody resulted in greater sensitivity to siderophore complexes with iron and other metals than to deferrated siderophore such that the quantities of siderophore measured primarily reflect the ferrated siderophore. Secondly, as in the earlier cited work with ferrioxamine B, the siderophore concentrations were diluted over the entire volume of rhizosphere soil that was extracted and would be much higher in the microsites containing actively growing bacteria where the siderophore diffuses out from microcolonies on the root surface. Using rough calculations to extrapolate the concentrations at the microsites of actively growing colonies, bacteria typically cover less than 1% of the total root surface area. As shown in Figure 8-3, if the actively growing bacteria are located along 10% of the root axes behind the root tips, then the siderophore concentrations would locally be 1000-fold higher or approximately 3 μM, which is a physiologically relevant concentration for growth of the bacterium.

3.2 Reporter gene technology for detection of siderophore production in the rhizosphere

With the advent of molecular biology techniques for study of the rhizosphere, recent studies on siderophore production soils have used reporter genes in which production of a marker protein is coupled to an iron regulated promoter to examine induction of the iron stress response system in bacterial cells. These techniques provide the advantage of being able to localize differences in iron stress responses, and have confirmed that siderophore production in the rhizosphere is highly dynamic in relation to differences in bacterial cell growth and the demand for iron in different root zones. This spatial variability was highlighted in studies using a genetically modified strain of *Pseudomonas syringae* containing a green fluorescent protein that was fused with an iron regulated promoter (Joyner and Lindow, 2000). When the bacterium was inoculated into soil containing bean plants, only about 10% of the cells in the rhizosphere showed high fluorescence intensity. Another independent approach to examine heterogeneity at the microsite level has been to inoculate soils with *Pseudomonas putida* containing an ice nucleation reporter gene that was fused with a promoter for the iron regulated operon for pyoverdin synthesis (Loper and Lindow, 1994). Experiments using this reporter system showed that coinoculation of soils with bacteria that produce other siderophores that can be used by the pseudomonad reduces the expression of the ice nucleation protein, whereas mutants that do not produce siderophores did not affect expression of the reporter gene (Loper and Henkels, 1999). Heterologous siderophores used by this particular pseudomonad included aerobactin and enterobactin. Thus the induction of siderophore production is influenced by the availability of iron from other siderophores and iron chelating substances that can be used by the pseudomonad. This principle is likely to be true for all siderophore producing microorganisms in the rhizosphere.

In addition to microbial siderophores, plant produced iron chelators have been shown to suppress the iron stress response of bacteria. In an experiment demonstrating this point, another pseudomonad, *P. fluorescens* 2-79 was modified with a plasmid containing the ice nucleation reporter and was used to examine changes in iron stress for cells of the bacterium grown in the rhizosphere of barley, rice, and lupin (Marschner *et al.*, 1997; Marschner and Crowley, 1998). In the experiments that were conducted with rice and barley, both plant species were grown in root boxes that allowed direct access to the plant roots where bacteria were lifted from the roots by blotting on to nitrocellulose filter papers. In this manner, the ice nucleation activity was calculated on a per cell basis and normalized to ice nuclei per 10^9 cfu of bacteria per gram of root. Replicate experimental units were compared in

which half of the plants had been supplied with a foliar iron spray to alleviate plant iron stress and thus shut down the plant iron stress response. Comparison of iron stress for pseudomonads in the rhizosphere of barley versus rice showed that the relative degree of iron stress was greater in the rice rhizosphere than in the barley rhizosphere, and that iron stress for bacteria on both plants was greater in plants that were treated with the foliar iron sprays.

The above results can be explained by the fact that the bacteria do not need to produce their own siderophores when the plant iron stress response functions to increase iron availability to the rhizosphere microorganisms. The iron stress response was greater in the rhizosphere of rice because barley produces higher quantities of phytosiderophores as compared to rice. With both plants, application of foliar iron shut down the plant iron stress response and the bacteria were forced to produce their own siderophores to acquire iron. To confirm this hypothesis, bacteria containing the ice nucleation reporter were grown in liquid media with iron supplied as ferric citrate and as phytosiderophores. The bacteria readily used both sources of iron and produced only small amounts of pseudobactin, and had low levels of ice nucleation activity. Similar results were obtained in a comparison of iron stress responses for cells grown on lupin, which does not produce phytosiderophores. In lupin, which produces high quantities of citric acid that are released under phosphorus stress conditions, iron stress for the bacteria containing the reporter gene was reduced under phosphorus limiting conditions when the plants produced high levels of citric acid. Conversely, application of phosphorus suppresses citric acid production and resulted in higher levels of iron stress. Altogether, these studies showed that Fe stress of *P. fluorescens* 2-79RLI in the rhizosphere may be influenced by plant species, P source, root zone and localization of the bacterial cells in different zones along the root axes.

3.3 Utilization of microbial siderophores by plants

Numerous studies have investigated the ability of plants to use microbial siderophores as iron sources for growth (Table 8-2), and have examined the possible mechanisms by which plants might obtain iron from these compounds, either by exchange with phytosiderophores or by the iron reductase that is expressed by Strategy I plants. However, these experiments have been controversial for several reasons. Since siderophores are labile and are subject to degradation after their addition to soil, almost all of the studies on plant use of microbial siderophores relied on hydroponic experiments in which radiolabeled siderophores were added to nutrient solutions at known concentrations generally ranging from 1 to 100 μM. Root

uptake rates and transport of iron to the plants shoots were then ascertained by measuring the quantity and distribution of iron in the plant. Iron provided by microbial siderophores is often found to be associated with the root tissues, which may be explained in part by uptake of iron by microorganisms that are associated with the root surface (Crowley *et al.*, 1992).

3.3.1 Strategy I plants

Iron uptake from siderophores by Strategy I plants has been examined for the hydroxamate siderophores ferrichrome, ferrioxamine B, rhodotorulic acid, and the mixed ligand siderophore pseudobactin produced by *Pseudomonas* spp. Other siderophores that have been studied and shown to be relatively effective for delivery of iron to plants include rhizoferrin, a citrate based siderophore produced by the fungus *Rhizopus*, and fusarinines, also produced by fungi. As a basis for comparison, iron uptake from siderophores is normally compared to that measured with supply of iron from synthetic chelates such as EDTA and HEDTA (Walter *et al.*, 1994; Johnson *et al.*, 2002). In general, iron acquisition from these compounds requires relatively high micromolar concentrations in hydroponic solutions where the entire root system is bathed in a uniform concentration of the siderophore. The utilization of siderophores that are used by extracellular reduction is sensitive to the presence of excess deferrated siderophore and to ferrous trapping agents such as BPDS (Yehuda *et al.*, 2000). This appears to be the most common mechanism with siderophores that have a high redox potential and that can be reduced to donate ferrous iron to the transport system of the plant. Ferrioxamine B on the other hand appears to be taken up directly and is only very slowly reduced by the Strategy I extracellular reductase (Wang *et al.*, 1993). With ferrichrome and ferrioxamine B, concentrations between 10 to 50 μM siderophore are required to support growth depending on the plant species.

The classical method for determining whether siderophores are used by reductive release of iron from iron chelates by Strategy I dicots involves the use of a ferrous trapping agent such as bipyridyl or BPDS which inhibit uptake of the ferrous iron that has been cleaved from ferric-ion-specific chelates and siderophores. Using these methods, reductase activity is minimal with siderophores such as ferrioxamine B, and only a small portion of the iron is translocated to the plant shoots within the short time frame over which these experiments are usually conducted (Crowley *et al.*, 1992; Johnson *et al.*, 2002). Other research using dual radiolabeled siderophore and iron has shown that in some cases plants may take up the intact iron-chelate, after which the entire complex eventually is transported to the shoot (Manthey *et al.*, 1996). It has been suggested that there may be a transport

system for this compound in some plants (Wang *et al.*, 1993; Johnson *et al.*, 2002). However, uptake might also occur *via* breaks in the apoplast at the sites of lateral root emergence where the siderophore iron complex may enter the xylem and undergo transport by mass flow in the transpiration stream to the plant leaves.

One interesting approach that was used to examine reductive removal or iron from siderophores and the role of root associated microorganisms employed a synthetically produced siderophore NBD-desferrioxamine B (NBD-DFO). This siderophore is an analog of the natural siderophore ferrioxamine B, but has the property of fluorescing when it is deferrated (Bar-Ness *et al.*, 1992). In this manner, the deferration process can be observed visually using a microscope. In experiments with this compound, iron uptake occurred by separation of iron from the chelate in the apoplast with no transport of the chelate across the cell membrane. Uptake rates were significantly affected by the presence or absence of root microflora, and was lower in the presence of an antibiotic agent, which suggests that some of the iron uptake was associated with use of the siderophore by rhizosphere microorganisms or perhaps that microorganisms reductively removed iron from the chelate which was then captured and transported by the plant iron transport system.

One of the most effective siderophores for supply of iron to plants is rhizoferrin, a siderophore produced by *Rhizopus arrhizus* (Yehuda *et al.*, 2000). This siderophore is readily reduced by the ferric chelate reductase of tomato and cucumber and supplies iron as effectively as the synthetic chelate EDTA. Rhizoferrin also supplies iron *via* ligand exchange to Strategy II monocots and supports the growth of corn and barley in iron deficient media. These findings are significant in that most studies on iron uptake mechanisms from siderophores have used only a few model compounds that have high affinity for iron. Overall, very few siderophores have been purified and used in experiments with plants, so it is possible that production and accumulation of siderophores such as rhizoferrin could contribute to plant iron uptake. However, more research is needed to determine whether the amounts of these low affinity siderophores may accumulate to concentrations that would support plant growth.

3.3.2 Strategy II plants

The primary mechanism by which iron is taken up by Strategy II monocots involves the production of phytosiderophores that are released into the rhizosphere to solubilize iron. Phytosiderophores are effective iron sources for some microorganisms and thus may suppress siderophore production of bacteria that use the compounds as alternative sources of iron.

On the other hand, there have been several studies that show that some microbial siderophores can mobilize iron from soils which can then undergo ligand exchange with phytosiderophores. The efficacy of this mechanism depends on the concentrations of the competing chelators, their stability constants for iron and other competing ions, the composition of the solution with respect to competing ions, and the pH and redox conditions in the root medium.

3.4 Inoculation of plants with siderophore producing microorganisms

The ability of plants to utilize microbial siderophores as iron sources for growth is highly controversial and has been investigated using a variety of experimental approaches using hydroponic and soil culture. Although there are considerable differences in the quantities of siderophores that are produced by various strains and species of bacteria, there is no evidence to date that inoculation with bacterial strains that produce high quantities of siderophores is beneficial to plant iron nutrition. Experiments in hydroponic culture show that inoculation of Strategy II plants with soil microorganisms can be detrimental to plants by causing rapid degradation of phytosiderophores which are not replaced by concomitant production of microbial siderophores at a commensurate level that can support plant growth (Duijff *et al.*, 1994; Walter *et al.*, 1994). This rapid degradation of phytosiderophores in hydroponics is thought to be an artifact of hydroponic culture where the microorganisms have rapid access to phytosiderophores that are released into the solution. In soils, the spatial separation of microorganisms from zones of high phytosiderophore release behind the root tip where bacterial populations are relatively low allows phytosiderophores to accumulate to locally high concentrations (Von Wirén *et al.*, 1993). Similar disappointments with inoculation have been observed in field experiments. In an experiment with two lines of oat, one capable of producing phytosiderophore and the second an iron inefficient cultivar, there was no benefit of inoculating the plants with siderophore producing bacterial strains (Alexander and Zuberer, 1993). In this case, the population densities of the siderophores producing strains were relatively low, ranging between 10^4 to 10^6 cells per gram of root. In this experiment, it was not clear whether the oat cultivars were inefficient in use of the microbial siderophores, or whether the population densities of the bacteria were too low to produce siderophores at concentrations that would be relevant to provide iron to the plants.

While plant iron nutrition may not be directly improved by soil inoculation with siderophore producing microorganisms, plant growth

promoting bacteria that stimulate plant growth through the production of hormones or destruction of plant produced ethylene rely on the use of siderophores for rhizosphere competence in iron-limiting soils (Forlani *et al.*, 1995; Bevivino *et al.*, 1998; Cattelan *et al.*, 1999). The enzyme, 1-aminocyclopropane-1-carboxylate deaminase (ACC), is responsible for the degradation of the precursor of ethylene which can inhibit plant growth. Bacteria that produce this enzyme also frequently produce siderophores. Auxins are produced by many different bacteria including *Enterobacter*, *Klebsiella,* and others (Haahtela *et al.*, 1990). These bacteria also produce siderophores which presumably increases their rhizosphere competence. In the search for bacteria and fungi that can be used as biofertilizers or for biocontrol of disease, siderophore production is commonly included as a criterion for characterizing potential soil and seed inoculants. However, it is clear that this trait alone does not necessarily confer plant-beneficial properties since many bacteria and fungi that are inhibitory to plant growth also produce siderophores (Gardner *et al.*, 1984; Kremer *et al.*, 1990).

Along with various bacteria that are being developed for biofertilizers, mycorrhizae are commonly advocated as soil inoculants in areas where severe disturbance has reduced the indigenous mycorrhizal inoculum in the soil. Superior mycorrhizal fungal strains are also being identified with the aim of using these as soil inoculants to enhance plant growth. The role of mycorrhizae for enhancing plant iron nutrition through siderophore production or utilization is still not clear, however. Mycorrhizal sorghum plants were shown to take up iron at higher concentrations than nonmycorrhizal plants (Caris *et al.*, 1998). It is still not clear whether the mycorrhizal fungi take up iron directly, or instead enhance the recovery of siderophores that are released by plant roots and their associated microflora. In the study by Caris and coworkers, mycorrhizae did not enhance iron uptake by peanuts, but did enhance iron uptake by sorghum, which produces phytosiderophores. It is thus of interest whether mycorrhizae are able to take up iron from the phytosiderophore-iron complexes and transport this iron to the plant roots for utilization by the plant.

4. ROLE OF SIDEROPHORES IN MICROBIAL INTERACTIONS

4.1 Competition between siderophores

The primary function of siderophores is to increase the fitness of bacteria in iron-limiting soils so that they can acquire sufficient amounts of this element for growth. Bacteria have many different functions in soils in which

they degrade plant detritus, fix nitrogen, detoxify potentially toxic chemicals, and interact with plants to promote plant growth or to cause plant diseases. Under highly competitive conditions, the ability to acquire iron via siderophores may determine the outcome of competition for different carbon sources that are available as a result of root exudation or rhizodeposition. Microorganisms can compete for iron based on differences in the quantities of siderophore that are produced, and the chemical properties of the siderophore with respect to it stability constant for iron. Other factors that come into play during competition include resistance to degradation which determines the potential accumulation of the siderophore, the ability to use siderophores that are produced by other microorganisms, and the specificity of the transport systems that are used for iron acquisition.

One of the possible ways in which siderophores may compete involves ligand exchange in which iron is stripped from a siderophore having low affinity for iron and is transferred to siderophores that bind iron more strongly. This is determined by the stability constant of the siderophore, which is dependent on the types of ligands that are used and their physical configuration as they coordinate around the metal center. Ligand exchange is a kinetic process that depends on the concentration of the siderophores in solution. This ability for chelates to undergo ligand exchange with iron has been measured between phytosiderophores and certain microbial siderophores with relatively low affinity for iron (Yehuda *et al.*, 1996; Hoerdt *et al.*, 2000), but is only speculated to occur between different types of microbial siderophores. The difference in stability constants for phytosiderophores and microbial siderophores is a key factor in controlling ligand exchange. Many microbial siderophores have very high stability constants for iron that are as much as 10 to 15 orders of magnitude greater. In the case where siderophores with high stability constants are present in mixtures with phytosiderophores, iron would be expected to undergo ligand exchange from phytosiderophores to microbial siderophores. The kinetics of ligand exchange may also be influenced by the chemical matrix and pH of the solution in which siderophore mixtures occur (Shenker *et al.*, 1996). Equilibrium models can help to predict the proportions of metals that are bound to different chelates, but kinetic processes may ultimately be more important in determining the extent to which different siderophores interact in competition for iron (Shenker *et al.*, 1999).

4.2 Siderophore mediated competition for iron and rhizosphere competence

Siderophores produced by fluorescent pseudomonads have extremely high binding efficiency for iron and are used in a strain specific manner such

that organisms that lack the specific transport systems for individual types of these iron chelators are unable to acquire iron. This extends not only to interactions between pseudomonads and pathogenic fungi, but also to interactions between different species and strains of pseudomonads. Insight into these interactions has been obtained largely from studies with mutant strains that are defective in either synthesis or transport of particular siderophores. These interactions are further complicated by the ability of pseudomonads to use heterologous siderophores via redundant siderophore production and transport systems (Raaijmakers *et al.*, 1995; Loper and Henkels, 1999). An example of where even subtle differences can affect competition was shown in studies with *Pseudomonas fluorescens* (C7R12), which was investigated in competition with a pyoverdine minus mutant of the same strain designated PL1, which was obtained by random insertion of the transposon Tn5 (Mirleau *et al.*, 2000). The mutant was significantly more susceptible to iron starvation than the wild-type strain despite its ability to produce another unknown siderophore. Both the wild type and the mutant PL1 were able to use pyoverdine and five other pyoverdines, although at a lower rate. In gnotobiotic model systems where either strain was grown separately, both the wild type and the mutant had similar survival in the rhizosphere, but when inoculated together, the wild-type predominated.

Pseudomonads are not alone in their ability to use heterologous siderophores, and it appears that the ability to use a variety of siderophore types can provide an advantage to rhizosphere microorganisms. Examples of the diversity of systems that can be carried by individual species of bacteria include *E. coli* where various strains have been isolated that have transport systems for enterobactin, ferrioxamine B, ferrichrome, and citrate or various combinations of the above. Another example is *Bradyrhizobium japonicum* which uses the hydroxamate-type siderophores ferrichrome and rhodotorulate, ferric citrate, and is also able to use pseudobactin (Plessner *et al.*, 1993). The advantage of being able to cross feed on different siderophores is nonetheless difficult to demonstrate as a consistent one. For example, a strain of *Pseudomonas* spp. carrying the plasmid pCUP2 which codes for a membrane receptor that allows use of ferric pseudobactin M114 was shown to be cross fed by a variety of related strains that produced similar types of pseudobactin (Moenne-Loccoz *et al.*, 1996). Despite the ability to cross feed, the fitness of this strain was not improved in comparison to the wild type that did not carry the additional siderophore receptor. One possibility is that the effects of competition require additional time to be manifested by differences in population size. This is illustrated by experiments examining competition between *Pseudomonas putida* WCS358 and *Pseudomonas fluorescens* WCS374 (Raaijmakers *et al.*, 1995) in which radish plants were

grown for four successive plant growth cycles after which the pseudobactin producing strain WCS358 had caused a 30 fold suppression of strain WCS374. However, in another treatment where WCS374 was genetically modified with a receptor protein for the pseudobactin produced by WCS358, it was able to maintain its population density. Despite this very clear example, other studies with strain WCS358 and indigenous bacteria that could use pseudobactin showed that there are still other competitive relationships that cannot be explained by competition for iron.

4.3 Siderophore involvement in suppression of plant pathogenic fungi

One of the most important functions of siderophores in mediating competitive interactions between microorganisms occurs in certain disease suppressive soils where fungal pathogens such as *Fusarium* and *Pythium* are inhibited by pseudomonads. Early research showed that production of large amounts of desferri-siderophore by pseudomonads in pure culture may sequester all available iron to the extent that they suppress growth of fungal pathogens that are cultivated on the same plate. Based on this observation, it was initially proposed that siderophores play a key role in antibiosis of plant pathogenic fungi. However, two decades of research on this topic have now provided detailed insight into the mechanisms of disease suppression. Some studies have provided direct evidence that siderophores have a direct role in the suppression of plant disease causing fungi (Duijff *et al.*, 1993). One strain in particular, *Pseudomonas fluorescens* WCS 358 discussed earlier has been shown to inhibit *Fusarium* primarily by siderophore mediated competition for iron (Leeman *et al.*, 1996a). In most cases, however, siderophores appear to be more important for contributing to the rhizosphere competence of disease suppressive bacteria than for causing iron deprivation to the pathogenic fungi. Effective strains of plant disease suppressive bacteria that suppress pathogenic fungi may use a variety of mechanisms in combination, including production of hydrogen cyanide, antibiotics, and lytic enzymes (Brisbane *et al.*, 1989; Pal *et al.*, 2000). Another mechanism by which bacteria can enhance disease suppression is by induction of systemic acquired resistance. This is stimulated by salicylic acid, which has the ability to chelate iron and is induced by iron stress. The pseudobactin produced by WCS374 may also induce the SAR response (Leeman *et al.*, 1996b). The discovery of this secondary effect of salicyclic acid and siderophores on the SAR response has led to reconsideration of the possible mode of action of siderophores for enhancing plant disease suppression.

Siderophore defective mutants of these disease suppressive bacteria sometimes have reduced ability to suppress fungal pathogens due to their impaired ability to acquire iron. However, as discussed in the section above, rhizosphere competence of disease suppressive bacteria can be enhanced by use of heterologous siderophores that are produced by other bacteria in the plant rhizosphere.

4.4 Siderophore production by symbiotic N_2 fixing bacteria

Iron is essential to the growth and nodulation of *Rhizobium* and is required in relatively high amounts by the nitrogen fixing bacteroids in root nodules to support nitrogen fixation. The nitrogenase enzyme system has a high iron requirement for synthesis of the iron sulfur centers in the enzyme complex. Iron is also required for the synthesis of leghemoglobin, which is used to deliver oxygen to the bacteroids. Because of their high demand for iron, strains of *Bradyrhizobium* that are capable of producing high quantities of siderophores are more efficient in nodulation than strains that are less iron efficient (Abd-Alla, 1999). On the other hand, the importance of siderophore production for survival of *Rhizobium* spp. in field soils is not as clear. In studies with *R. meliloti* there were no apparent differences in the relative abundance of *Rhizobium* lines with low and high siderophore production capabilities (Barran and Bromfield, 1993). In another study, strains of *Bradyrhizobium* were engineered to overproduce siderophore by insertion of a transposon and were then tested to determine their survival and nodule occupancy (Manjanatha *et al.*, 1992). Strains that overproduced siderophore had lower nodule occupancy than the wild type, which suggested that iron was not limiting for the wild type and there was no advantage to overproduction. However, there may also have been other unintended effects of the transposon mutagenesis that lowered the competitive abilities of the overproducing strains.

Pseudomonads have been found to enhance the growth of clover and symbiotic nitrogen fixation by *Rhizobium leguminosarum* sp under gnotobiotic conditions (Marek-Kozaczuk *et al.*, 1996). To further examine whether this was associated with iron, siderophore defective mutants of the pseudomonads were constructed and inoculated into the rhizosphere. In this case, there was no difference between the wild type and the sid- mutants, suggesting that the growth promotion effects were not related to iron availability.

5. CONCLUSIONS

Microbial siderophores play a key role in facilitating the uptake of iron by rhizosphere microorganisms in iron limiting soils. Iron limitation occurs primarily under aerated conditions at alkaline pH where siderophores are required to mobilize iron by dissolution of solid phase minerals. Almost all microorganisms either produce some type of siderophore or are able to use generic siderophores produced by various microorganisms in their vicinity. In addition to solid phase iron minerals, organic matter and plant detritus can serve as potential sources of iron that can be mobilized by siderophores. In the plant rhizosphere, the iron stress response of microorganisms is affected also by plant iron stress responses that are induced to mobilize iron. Phytosiderophores and citrate are readily used by many different microorganisms for iron acquisition, and microorganisms appear to rely on their own siderophore transport systems to the extent necessary for supplementing iron that has been mobilized by plant iron stress responses.

A few different siderophores have been shown to serve as iron sources for plants, and are used by ferrochelate reductase which reductively cleaves ferrous iron from ferric iron specific siderophores. However, this appears to be effective only for certain siderophores with relatively low affinity for iron, and common siderophores such as ferrioxamines and pseudobactins are poorly used by this mechanism. In general, the amounts of microbial siderophore that are produced in the rhizosphere appear to be too low to be of major significance for plant iron nutrition. However, detection methods for siderophores limit the ability to quantitatively measure localized siderophore concentrations in the rhizosphere. The use of reporter gene technology suggests that only a small portion of bacterial cells in the rhizosphere are growing at rates where iron demand is sufficient to induce siderophore production. The direct role of bacterial siderophores in causing suppression of plant pathogenic fungi by iron deprivation is now being re-evaluated and appears to be only one component of disease suppression. Nonetheless, production of siderophores provides an advantage to bacterial species that are inoculated into soils for biocontrol. As microorganisms are increasingly being studied for their potential ability to promote plant growth and for biocontrol of plant diseases, microbiologists will need to continue to consider the fundamental role of siderophores in the ecology of the plant rhizosphere.

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Chapter 9

THE METABOLISM OF IRON BY NITROGEN-FIXING RHIZOSPHERIC BACTERIA

LARRY L. BARTON, GORDON V. JOHNSON and YVONNE M. BISHOP

Department of Biology, University of New Mexico, MSC03 2020, Albuquerque, New Mexico 87131-0001, USA. E-mail: lbarton@unm.edu

Abstract: Nitrogen-fixing bacteria require iron for rhizospheric growth as well as for plant-associated symbiotic activities. One of the iron uptake systems involves siderophores and bacteria produce a great diversity of ferric ion chelators in iron-limited environments. This review examines some of the free-living nitrogen-fixing bacteria, including *Azotobacter vinelandii* which produces three different examples of iron-binding catecholates. *Frankia* and bacteria of the rhizobial group also produce siderophores and many of the rhizobia are capable of using different forms of chelated iron. When grown in pure culture, *Frankia* and rhizobia produce siderophores; however, when grown symbiotically the production of siderophores is not fully resolved. The growth of symbiotic bacteria *in planta* is highly complex and this review examines some of the metabolic activities of iron in this environment. While the hemoglobin in the nodules of plants promotes bacterial metabolism, there are numerous details that need clarification with respect to cellular regulation in a low oxygen environment. Additionally, this review calls attention to the oxygen stress response that may be attributed to oxidation of non-chelated ferric ions which could be a consequence of siderophores with a low iron affinity.

Key words: nitrogen fixation; rhizobia; rhizospheric bacteria; root nodules; siderophores

1. INTRODUCTION

As described by Fraústo da Silva and Williams (2001) cell composition can be divided into three states: the metallome which reflects the metallic element content, the proteome which includes cell proteins, and the genome which encompasses cellular DNA. Recently the term ionome (Salt, 2004)

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has been used to include anions and cations of metals and metalloids found in biological systems. For rhizospheric organisms including the nitrogen-fixing bacteria, Fe is one of these essential elements of the ionome, or more precisely metallo-ionome. Prokaryotic nitrogen fixation is an activity of paramount importance because the quantity of molecular nitrogen converted to ammonia occurs at a rate of 170 x 10^{12} g yr^{-1} which exceeds the rate for industrial conversion and natural atmospheric fixation of nitrogen (Taiz and Zeiger, 1998). Nitrogen-fixing bacteria not only require Fe for electron shuttle reactions and enzymatic activities but also require Fe as an important element for nitrogenase activity. The metabolic requirement for elements of the metallome reflects the environment where cells are found and for rhizospheric bacteria the intracellular region or cytoplasm and the extracellular or soil matrix are the environments of interest. However, for some nitrogen-fixing bacteria there is an additional environment and it is in the root nodule, where bacteria become differentiated as it grows symbiotically with the plant. This review will examine the metabolism of Fe by nitrogen-fixing bacteria in these various environments.

2. THE RHIZOSPHERIC ENVIRONMENT AND IRON CHELATES

2.1 Rhizobia-symbiotic nitrogen-fixing bacteria

The group of bacteria collectively referred to as rhizobia, with one known exception, use leguminous plant roots as the host. The taxonomy of the Gram-negative rhizobia is not resolved; however, for the purpose of illustration a list of some of these bacteria along with their plant hosts are given in Table 9-1 (Van Berkum and Eardly, 1998). In some cases the bacteria listed in Table 9-1 may nodulate additional species and the reader is referred to the publication of Hadri *et al.* (1998) concerning alternate hosts.

The Fe nutrition of root nodule bacteria has attracted the attention of many scientists and results indicate that considerable variation exists in the response of different strains to Fe deficiency. Rhizobia require Fe and phosphorous to grow in the rhizosphere and to nodulate roots of legumes (O'Hara, 2001). Tang *et al.* (1992) have reviewed investigations on the response of the legume symbiosis to Fe deficiency. Additionally, Fe, calcium, phosphorus, cobalt, copper, potassium, nickel, selenium, zinc, boron, and molybdenum are important for nodule development and nitrogen fixation. With respect to Fe utilization, rhizobia have been found to utilize $FeCl_3$, Fe-citrate, synthetic Fe-chelates, siderophores, and even heme. A listing of some of the various biochelates utilized by rhizospheric bacteria

including rhizobia is given in Table 9-2 and additional siderophores for rhizobia are described in an earlier review (Barton *et al.*, 1994).

Table 9-1. Examples of plants with nodules attributed to rhizobia.

Bacterial symbiont	Plant host	Common name or description
Azorhizobium caulinodan	*Sesbania rostrata*	aquatic tropical legume
Bradyrhizobium sp.	*Parasponia* (formerly *Trema*)	a non-legume
Bradyrhizobium japonicum	*Glycine max* (L.) Merrill	soybean
Bradyrhizobium sp.*	*Aeschynomene indica*	aquatic plant with adventitious roots
Mesorhizobium ciceri	*Cicer arietinum* (L.)	chick pea
Mesorhizobium loti	*Lotus corniculatus*	lotus
Rhizobium etli	*Phaseolus vulgaris* (L.)	bean
Rhizobium tropicli	*Phaseolus vulgaris* (L.)	bean
Rhizobium leguminosarum bv *phaseoli*	*Phaseolus vulgaris* (L.)	bean
Rhizobium leguminosarum bv *trifoli*	*Trifolium repens* (L.)	clover
Rhizobium leguminosarum bv *viciae*	*Pisum sativum* (L.)	pea
Sinorhizobium meliloti	*Medicago sativa*	alfalfa

*It has been proposed that this pigmented strain be *Photorhizobium*, a new genus.

Relatively few strains of *Bradyrhizobium* have been demonstrated to produce siderophores when using traditional chemical assays. In a survey of several strains Carson *et al.* (2000) did not find any that produced siderophores and Guerinot *et al.*, (1990) found that citrate was the only siderophore produced by one of 20 strains of *Bradyrhizobium japonicum*. The examination of 18 strains of *Bradyrhizobium* nodulating groundnut using the chrome azurol S (CAS) reagent (Schwyn and Neilands, 1987) revealed only small halos around colonies on the blue agar plates. In a liquid CAS assay, it appeared that either only a very low level of siderophore was produced by these strains when under Fe stress conditions or that the Fe chelators had a relatively low affinity for Fe^{3+} (Van Rossum *et al.*, 1994). In a separate study, Abd-Alla (1999) found that only two out of six strains of *Bradyrhizobium* spp. effective in nodulating lupin produced a hydroxamate-type siderophore when grown under Fe-stress conditions and that mannitol proved to be the best carbon source for this siderophore production.

In addition to Fe-citrate utilization, strains of *Bradyrhizobium japonicum* have been reported to use ferrichrome, rhodotorulate, and pseudobactin which are siderophores produced by microorganisms other than rhizobia (Plessner *et al.*, 1993). Both *Bradyrhizobium japonicum* and *Rhizobium leguminosarum* bv *viciae* have an Fe uptake system that uses heme supplied as hemoglobin or leghemoglobin; however, for these organisms, heme is an alternative Fe supply system for free-living bacteria and is not required for nitrogen-fixation (Nienaber *et al.*, 2001; Wexler *et al.*, 2001). The heme

uptake system is under the regulation of Fur and consists of 9 genes including a heme receptor in the outer membrane, a periplasmic binding protein and an ATP driven ABC transporter in the plasma membrane. To energize the uptake of heme, a TonB homologue protein extends from the plasma membrane to the outer membrane (Wexler *et al.*, 2001). Fur does not control the production of this TonB homologue even though TonB-like protein is reduced by Fe starvation. LeVier and Guerinot (1996) have reported an outer membrane protein, FegA, of 80 kDa with a sequence similar to Ton-B dependent proteins from other bacteria. Production of FegA is under the influence of Fe starvation and this protein appears structurally similar to protein receptors for the hydroxamate class of siderophores.

Table 9-2. A selection of siderophores used by rhizospheric bacteria.

Bacteria	Iron uptake system	Siderophore	Reference
Azotobacter vinelandii	protochelin	tricatecholate	1
	azotocholate	dicatecholate	1
	aminochelin	monocatecholate	1
Azospirillum brasilense	spirillobactin	hydroxamate	2
Azospirillum lipoferum	dihydroxybenzoic acid	hydroxamate	3
Bradyrhizobium japonicum	heme	not applicable	4
Frankia sp.	frankobactin	hydroxamate	5
Rhizobium leguminosarum bv. phaseoli	not specified	hydroxamate	6
Rhizobium leguminosarum bv. viciae	vicibactin	trihydroxamate	7
Rhizobium leguminosarum bv. trifolii	vicibactin	trihydroxamate	8
Sinorhizobium meliloti	rhizobactin 1021	citrate derivative	9

1. Cornish and Page, 1998. 2. Bachhawat and Ghosh, 1987. 3. Saxena *et al.*, 1986. 4. Nienaber *et al.*, 2001. 5. Boyer *et al.*, 1999. 6. Carrillo-Castaneda and Cano, 2000. 7. Dilworth *et al.*, 1998. 8. Lynch *et al.*, 2001, 9. Persmark *et al.*, 1993.

The structure of the siderophore produced by *Sinorhizobium meliloti* strain 1021 was established by Persmark *et al.* (1993). It is a citrate derivative containing two diaminopropane moieties and this siderophore has been designated rhizobactin 1021. Other strains known to produce rhizobactin include strains 2011 and SU47. The production of rhizobactin 1021 by *Sinorhizobium meliloti* strain 2011 results from a cluster of 8 genes found on a megaplasmid and these genes function for regulation, biosynthesis, and transport (Lynch *et al.*, 2001). From analysis of the 6 genes (rhbABCDEF) required for biosynthesis of rhizobactin 1021, it appears that initially the 1, 3-diaminopropane structures are produced and subsequently, the diamino-propane moieties are attached to citrate.

2.2 Frankia-actinorhizal root nodule symbiosis

Frankia is an actinorhizal bacterium that nodulates roots of woody shrubs or trees and a partial listing of plants with *Frankia* are given in Table 9-3. Nodules attributed to *Frankia* have been reported for eight families and 25 genera of dicotyledonous plants. All of these nodule bacteria are collectively referred to as *Frankia* with species designations to be established after further molecular analysis. An excellent summation of the activities of *Frankia* is given in the book edited by Schwintzer and Tjepkema (1990). The nitrogenase activity is found in thick walled vesicles that are found on short branches extending from the bacterial mycelium and the rate of nitrogen fixation by nodules containing *Frankia* is comparable to that of legume nodules with rhizobia. Unlike rhizobia cultures, some *Frankia* isolates can be adapted in culture to readily fix dinitrogen under aerobic conditions.

Relatively few strains of *Frankia* have been found to produce siderophores. Siderophores of the catechol class were produced by four isolates from roots of members of the *Casuarinaceae* (Arahou *et al.*, 1998). However, *Frankia* strain 52065 which was isolated from *Ceanothus americanus* produces a hydroxamate siderophore when cultivated under Fe limited conditions of growth (Boyer *et al.*, 1999). Siderophores from *Frankia* have been termed frankobactin and *Frankia* strain 52065 produces two siderophores: frankobactin and frankobactin A. The structural difference of these siderophores is that the phenyl oxazoline ring in frankobactin is closed while in frankobactin A the ring is open (Boyer *et al.*, 1999). The status of the oxazoline ring accounting for two structural types of siderophores is not novel for frankobactin because similar dual structures of agrobactin and agrobactin A are reported for *Agrobacterium tumefaciens* (Ong *et al.*, 1979), while parabactin and parabactin A have been reported for *Paracoccus denitrificans* (Peterson *et al.*, 1980). Both frankobactin and frankobactin A are produced simultaneously by *Frankia* strain 52065 in rapidly growing cultures; however, these experiments are not for the impatient because the lag phase for an Fe-limited culture is about 30 days (Boyer *et al.*, 1999).

When following the uptake of ^{55}Fe, Boyer *et al.* (1999) observed that *Frankia* strain 52065 showed greater preference for ferric loaded desferal or frankobactin than for citrate, EDTA, or pseudobactin. Since desferal is a derivative of ferrioxamine B, the main siderophore of *Streptomyces pilosus*, ferrioxamine B was also tested and the rate of ^{55}Fe uptake with both of these siderophores was comparable. The uptake of frankobactin by *Frankia* is by an energy-requiring mechanism (Boyer and Aronson, 1994).

Symbiotic bacteria that are not covered in this review include the cyanobacteria *Anabaena* and *Nostoc* that grow with *Azolla* and *Gunnera*, respectively. The reader is referred to a publication by Wilheim (1995) for physiological responses of cyanobacteria to Fe-limited growth.

Table 9-3. A selection of perennial dicots producing nodules with *Frankia.*

Family	Genus	Common name
Betulaceae	*Alnus*	Alder
Casuarinaceae	*Allocasuarina*	Black she-oak
	Casuarina	Beef wood
	Gymnostoma	Daintree
Coriariaceae	*Coriaria*	a low growing shrub
Datiscaceae	*Datisca*	Durango root
Elaeagnaceae	*Elaeagnus*	Russian olive, Autumn olive
	Hippophae	Sea buckthorn
	Shepherdia	Buffalo berry
Myricaceae	*Comptonia*	Sweet fern
	Myrica	Bog myrtle
Rhamnaceae	*Ceanothus*	Buckbrush, Wild lilac
	Colletia	Anchor plants
	Discaria	Matagouri, Tumatakuru
	Retanilla	No common name
	Talguenea	Talguen
	Trevoa	Trevo bush
Rosaceae	*Cercocarpus*	Mountain mahogany
	Cowania	Cliffrose
	Purshia	Desert bitterbrush

2.3 Free-living and plant-associated bacteria

Due to the low solubility of Fe in soils, nitrogen-fixing bacteria have acquired several different strategies for the acquisition of Fe from the environment. Since the utilization of Fe by rhizospheric bacteria has been discussed in Chapter 8, this chapter will focus on the nitrogen-fixing bacteria. Several species of rhizobacteria are commonly found associated with plant roots where they derive benefit from organic compounds released from the root and enhance plant growth through nitrogen fixation. Organisms of the genus *Azospirillum* are important plant growth promoting bacteria that are colonized principally on the root surface and have been extensively characterized (Steenhoudt and Vanderleyden, 2000). Several endophytic diazotrophic bacteria are found and the ones most commonly isolated include *Acetobacter diazotrophicus*, *Herbaspirillum seropedicea*, and *Azoarcus* sp. (Elmerich *et al.*, 1992). Iron-uptake activities of the associated bacteria other than *Azospirillum* have not been established.

Under Fe limited conditions, the uptake of Fe from the soil by *Azospirillum* is attributed to siderophores (see Table 9-2). Derivatives of

dihydroxybenzoic acid (DHBA) are produced as siderophores of *Azospirillum* with *Azospirillum lipoferum* producing 2, 3-DHBA and 3, 5-DHBA leucine and lysine conjugates. The production of 3, 5-DHBA in *Azospirillum lipoferum* appears to be regulated by both Fe^{3+} and molybdate because production of this siderophore is increased under molybdate as well as Fe starvation (Saxena *et al.*, 1989).

Azotobacter vinelandii produces several catecholate siderophores (Table 9-2) and these molecules may have multiple roles in bacterial and plant nutrition. Of considerable interest is the observation that the catecholate siderophore forms complexes with Mo^{5+} as well as with Fe^{3+} (Duhme-Klair, 2003; Duhme-Klair *et al.*, 2003). Not only do molybdate and transition metals have a role in accumulation of protochelin by *Azotobacter vinelandii* (Cornish and Page, 2000) but also a siderophore derivative serves as a selective signal for molybdate (Jedner *et al.*, 2001). The ferri-siderophores, loaded with Fe, of *Azotobacter vinelandii* are used by *Agrobacterium tumefaciens* and *Erwinia carotovora* (Page and Dale, 1986). Thus, the *Azotobacter* siderophores, unlike those of *Pseudomonas putida* (Leong and Expert, 1989), do not inhibit phytopathogenic bacteria. It has been reported that *Azotobacter* enhances the growth response of oat (Singh *et al.*, 2000) and the shelf-life of tomato (Chaurasia *et al.*, 2001). It would be important to learn if siderophores produced by *Azotobacter* have a role in each of these activities.

3. FERRIC REDUCTASE AND FERRITIN

Widely distributed throughout various genera of bacteria is an enzyme that reduces Fe^{3+} to Fe^{2+} and it is commonly referred to as ferric chelate reductase or assimilatory ferric reductase. As reviewed by Schroder *et al.*, (2003), the ferric ion reductases from microorganisms are in fact flavin reductases where the flavin is not covalently bonded to the protein but is required for reduction of ferric ion. Most commonly the reductase is found in the plasma membrane or in the cytoplasm where the enzyme is involved in specific metabolic roles. It may be assumed that most bacteria have an enzyme that reduces ferric ion and this enzyme would be important for acquisition of Fe from different types of Fe chelates. Therefore, even though ferric chelate reductase has been reported for relatively few rhizobia (Barton *et al.*, 1992) it is expected to be present in symbiotic nitrogen-fixing bacteria. With *Azotobacter vinelandii*, the inhibition of ferric reductase by Zn^{2+} or Mn^{2+} signals hyperproduction of azotobactin (Page, 1995).

There has been some suggestion that rhizobia store Fe. Using cultures of *Sinorhizobium meliloti*, Gill *et al.* (1991) reported that two serial passages

into Fe-free growth medium is required to deplete Fe stores in rhizobia. This storage of Fe in rhizobia could be attributed to the presence of either ferritin or bacterioferritin (Andrews, 1998). In highly aerated cultures growing in an Fe rich medium, bacteria will acquire and store Fe in these unique structures. While the ferritin in bacteria is remarkably similar to eukaryotic ferritin, bacterioferritin has a heme moiety attached to the subunits making up the ferritin structure. An obvious function of ferritin in bacteria is to deplete free ionic Fe from the cytoplasm and store it as ferric ion in specific Fe-containing molecular structures. It is not desirable for free Fe^{3+} to be present in the cytoplasm because interaction with O_2 could result in the formation of highly toxic free radicals. A role of the heme groups attached to the ferritin unit in bacteria is to aid in the oxidation–reduction reactions moving Fe into and out of ferritin. An additional role of bacterioferritin may be to regulate the redox state in the cytoplasm. While ferritin or bacterioferrritin have never been reported for rhizobia, *Frankia*, or *Azotobacter* it is highly probable that some type of Fe storage unit would be present in these bacteria.

Thus, in Fe storage in symbiotic bacteria the following series of events can be proposed:

- Aerobic growth of bacteria in the rhizosphere would lead to intracellular storage of Fe.
- In the establishment of a root nodule, Fe would be withdrawn from the ferritin system.
- By the time an anaerobic region in the nodule is established, bacteroids would be depleted of stored Fe in ferritin or bacterioferritin and the heme moieties of bacterioferritin would help poise the intracellular redox state.

Future studies will be needed to evaluate the presence and possible function of an Fe storage system in rhizobia.

4. IRON METABOLISM IN THE NODULE

One of the features of nodule metabolism that has been examined is the extent that siderophores are required for differentiation of symbiotic rhizobia into bacteroids or for nitrogen fixation. It has been reported that Fe is required for optimum nodulation and development of bacteroids in the nodules on peanut plants (Tang *et al.*, 1992; O'Hara *et al.*, 1988). Studies addressing the requirement of siderophores for symbiotic nitrogen fixation have involved molecular biology studies, nitrogen fixation assays, and plant yield measurements.

To evaluate if siderophores are required for the symbiotic rhizobia to fix nitrogen, Gill and Neilands (1989) used Tn5 mutagenesis with *Sinorhizobium*

meliloti strain 2011 to generate an interesting set of cultures. Bacterial mutants had the following limitations: defective in production of rhizobactin 1021, defective in uptake of rhizobactin 1021, or lacking transcriptional control in regulating production of rhizobactin 1021. While there was nitrogen fixation by the three classes of mutants and by wild-type *Sinorhizobium meliloti* strain 2011, greatest level of nitrogen fixation activity as assessed by a variety of criteria was with the wild-type culture (Gill *et al.*, 1991; Barton *et al.*, 1992). However, molecular experiments evaluating mRNA produced in the mature nodule containing *Sinorhizobium meliloti* (Lynch *et al.*, 2001) revealed that genes for siderophore production or transport were not strongly expressed when genes for nitrogen fixation were transcribed.

Siderophore production by rhizobia appears to be required for high plant yield. In an interest of selecting the best strains of *Bradyrhizobium japonicum* for inoculation of soybeans, Khandelwal *et al.* (2002) conducted pot studies and field trials with several different bacterial strains. When plants were inoculated with a bacterial strain producing a siderophore, higher soybean yields were observed when compared to six bacterial strains not producing an Fe chelator. This increased plant performance was observed in chlorophyll content, number of nodules, root length, shoot length, and number of soybean pods per plants. In a related study, it was determined that a strain of *Sinorhizobium meliloti* efficient in acquisition of Fe would out-compete strains of bacteria that had low intracellular Fe levels due to Fe limited growth or to inability to acquire Fe from the medium (Battistoni *et al.*, 2002).

While plant roots contain low levels of hemoglobin, there is an increase in the level of hemoglobin in bacterial nodules. Hemoglobin levels in *Frankia* nodules with *Myrica* or *Casuarina* as hosts is similar to that found in legume nodules established by rhizobia; however, hemoglobin in many of the host plants for *Frankia* is relatively low (Silvester *et al.*, 1990). Using microelectrode measurements, pO_2 in the actinorhizal nodules containing the central infected zone is < 1 kPa O_2 while the oxygen level in the nodule cortex is at the atmospheric level. Since *Frankia*, unlike rhizobia, can adapt to fix nitrogen in the presence of oxygen, the role of hemoglobin may be to carry O_2 into the actinorhizal nodule where there is a diffusion barrier due to a thick cell wall surrounding the nodule. However, in many nodules, the level of hemoglobin is low and the role of hemoglobin in these actinorhizal nodules remains unknown.

An intriguing feature of unknown relevance is that *Frankia* produces an intracellular hemoglobin molecule (Tjepkema *et al.*, 2002). When cultivated *ex planta*, *Frankia* strain Ce13 produces a truncated hemoglobin with a molecular weight of 14.1 kDa and this molecule is 20 to 40 amino acids

smaller than the classical hemoglobins. While the function of this hemoglobin is unresolved, hemoglobin from *Frankia* has a high binding affinity for O_2 as well as for CO. Prokaryotes that produce hemoglobin in addition to *Frankia* includes *Mycobacteria*, *Synechocystis*, and *Nostoc.* Hemoglobin found in actinorhizal nodules of *Casuarina* is not produced by *Frankia* but appears to be from the plant host.

Iron is an important metal in rhizobial nodules because of the level of hemoglobin in the nodule and Fe-containing enzymes in the bacteria. Leghemoglobin represents 20% to 30% of the soluble protein in the legume nodule while nitrogenase is 10% to 12% of the soluble protein in rhizobia (Verma and Long, 1983). Leghemoglobin occurs in both the soybean cell cytoplasm (~3 mM) and within the region surrounded by the peribacteroid membrane it is found at 200-500 nM (Appleby, 1984). The source of leghemoglobin is unresolved with the possibility that both protein and heme is produced by the plant or that leghemoglobin is a true symbiotic protein with heme produced by the bacteria and globulin produced by the plant. Using a mutant of *Sinorhizobium meliloti* defective in heme synthesis, Legon *et al.* (1982) reported that nodules lacked leghemoglobin while Guerinot and Chelm (1986) found that nodules resulting from a heme mutant of *Bradyrhizobium japonicum* did produce leghemoglobin. The role of leghemoglobin appears to be associated with transporting oxygen to the bacteroids for rhizobial respiration (Appleby, 1984). In nodules with active nitrogenase, 30-48% of the leghemoglobin is oxygenated and the O_2 concentration is 17-30 nM while nodules where the nitrogenase is markedly inhibited, 11-17% leghemoglobin is oxygenated and the O_2 concentration is 5-8 nM (Werner, 1992). At low levels of oxygen respiration of bacteroids is selectively supported because the affinity of bacteroids for O_2 is 5 nM while the cytochromes in mitochondria have an affinity of 100 nM for molecular O_2.

In soybean root nodules, differentiated rhizobia cells are referred to as bacteroids and these bacteroids are enclosed by a peribacteroid membrane that collectively forms symbiosomes. The transport of Fe chelates across the peribacteroid membrane is at a faster rate than the transport across the bacteroid membrane resulting in an Fe storage pool in the peribacteroid space (LeVier *et al.*, 1996). The amount of non-leghemoglobin Fe present in the peribacteroid space is on the order of 0.5 to 2.5 mM and this Fe has been reported to be bound by siderophores produced by the bacteroids (Wittenberg *et al.*, 1996). Ferric citrate is transported across the peribacteroid membrane and the bacteroid membrane (Moreau *et al.*, 1995). Le Vier *et al.* (1996) found that Fe supplied as chelates of several other organic acids was transported across both membranes and in addition detected ferric citrate reductase activity at the peribacteroid membrane. This suggests the model

including both transport of ferric chelates and also a reductive mechanism (Udvardi and Day, 1997). If indeed the peribacteroid membrane is produced from the plant cell membrane, ferric chelate reductase in the peribacteroid membrane would be expected to be similar to the ferric reductase in the plant cell membrane.

Thus, several steps in Fe metabolism would be expected to occur as Fe moves from the rhizosphere into the bacteroids in the nodules. The following reactions are proposed for Fe transport in the bacterial nodules:

- At the exterior of the peribacteroid membrane, Fe would be reduced from Fe^{3+} to Fe^{2+} by a membrane bound ferric reductase requiring NADH.
- The Fe^{2+} is oxidized in the peribacteroid space by an unspecified reaction.
- The ferrisiderophore binds onto a protein on the surface (outer membrane) of the bacteroid and the ferrisiderophore is transported across the plasma membrane into the cytoplasm of the bacteroid.
- Alternatively, Fe^{2+} may be released from the ferrisiderophore by a ferric chelate reductase in the plasma membrane of the bacteroid and Fe^{2+} may be transported across the plasma membrane by a divalent cation transporter.

Although this shuttle system for Fe in the nodule requires additional characterization, an efficient Fe transport in the nodules would promote a high capacity to nodulate biomass, nitrogen fixation, and plant biomass. This could account for greater yield of beans (Krouma and Abdelly, 2003; Khandelwal *et al.*, 2002) observed with siderophore-producing bacteria as compared to the use of non-siderophore producing strains as inoculum.

5. IRON AND OXYGEN TOXICITY

Just as maintaining a proper level of Fe in plants has become an important topic, Fe homeostasis is important for nitrogen-fixing bacteria. The unique physiological state of nitrogen fixation by rhizobia requires a very low intracellular oxygen level because NifA is irreversibly inactivated with O_2 (Barbour *et al.*, 1992). In *Azospirillum lipoferum* and *Azospirillum brasilense*, nitrogenase is controlled by NifA which is inactivated by high intracellular levels of oxygen and nitrogen compounds. In *Sinorhizobium meliloti*, the low concentration of oxygen in the nodule signals the expression of the *nif* and *fix* genes for nitrogenase production. Bacteroids isolated from nodules attributed to *Rhizobium leguminosarum* and to *Bradyrhizobium japonicum* have optimum nitrogenase activities at 800 nM O_2 and 100 nM O_2, respectively (Kaminski *et al.*, 1998). Oxygen metabolism by the symbiosome is critical because oxygen-dependent respiration by the

bacteroids is required to provide the 15-18 ATP for each N_2 molecule fixed; however, oxygen toxicity is an additional concern and bacterial systems usually have an elaborate system involving oxygen stress response. Thus, there is the paradox in the nodule of having sufficient O_2 but not having an excess which would be both inhibitory for gene expression or enzyme structure and toxicity attributed to the formation of free radicals.

A consequence of Fe^{3+} presence is the interaction with molecular oxygen to produce toxic free radicals. The catecholate siderophores of *Azotobacter vinelandii* are important in management of oxidative stress because protochelin and azotochelin, but not aminochelin, were able to prevent the generation of the hydroxyl free radical by a series of reactions involving free Fe^{3+} (Cornish and Page, 1998). Reactions leading to hydroxyl radical formation are as follows:

(Iron reduction) $O_2^- + Fe^{3+} \longrightarrow Fe^{2+} + O_2$
(Fenton's reaction) $Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH^{\cdot}$

The low binding affinity of aminochelin for Fe^{3+} is reflected by the ligand:Fe^{3+} ratio for protochelin, azotochelin and aminochelin which was determined to be 1:1, 3:2, and 3:1, respectively. Perhaps other siderophores which do not readily give a positive CAS response also do not bind Fe^{3+} with sufficiently affinity to prevent the formation of free radicals. The regulation of transcription of siderophore genes in *Azotobacter vinelandii* (Tindale *et al.*, 2000) by the oxidative stress response could reflect a cell response mechanism to prevent oxidative damage in Fe-limited cells.

6. PERSPECTIVE

Considerable progress has been made to understand siderophore production by the different rhizobia species and by *Azotobacter*. Current research is moving in the direction of integrating the various aspects of bacterial Fe metabolism with the myriad of cellular activities in the free living organism as well as in the root-associated nodule. In the nodulating and free-living bacteria, the genes for Fe uptake as well as for nitrogen-fixation are regulated, in part, by readily available Fe and nitrogen in the extracellular environment. However, it is now apparent that oxygen concentrations signal gene expression for these metabolic systems. Of concern is the potential for free ferric ion to contribute to the oxidative stress activity. As research progresses in the area of Fe uptake and utilization, attention should also focus on the multiple component signaling systems involved. Future experiments addressing the sensory regulation of Fe

metabolism will require the contribution of many laboratories and the summation of their findings are sure to provide a most interesting contribution.

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Chapter 10

GENETIC REGULATION OF IRON IN *Erwinia chrysanthemi* AS PERTAINS TO BACTERIAL VIRULENCE

DOMINIQUE EXPERT
Laboratory of Plant Pathology (CNRS-INRA), Paris Grignon 16 rue Claude Bernard, 75231 Paris Cedex 05, France. E-mail: Dominique.Expert@inapg.inra.fr

Abstract: Plants represent privileged habitats for many microorganisms because they are a prime source of nutrients. In particular, they are exposed to pathogenic microorganisms causing a variety of diseases that may be economically important if environmental conditions are permissive. Elucidating the mechanisms of microbial virulence and plant immunity at the physiological and molecular levels should contribute to the development of integrative strategies for controlling plant diseases. A pathogenic bacterium produces virulence factors enabling it to gain access to nutrients and to escape host defenses. In this regard, the capacity of a pathogen to acquire iron in the host plant may be an important issue because this metal is essential for the metabolism but however not readily available in biological tissues. Iron has long been reported as a clue to bacterial virulence and animal host defense. This question has been a major concern of our research work focused on the soft rot disease caused by *Erwinia chrysanthemi,* a pathogen attacking a wide range of plants of agricultural importance. We have addressed different questions: what are the mechanisms of iron acquisition and homeostasis involved in *E. chrysanthemi*, during pathogenesis? Does iron availability act as a triggering signal for expression of virulence factors? Are specific plant reactions induced that lead to iron deprivation of the bacterium upon infection? This chapter illustrates these questions and shows how *E. chrysanthemi* adapts its iron metabolism to stressing conditions encountered in the host.

Key words: bacterial virulence; iron homeostasis; siderophore

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1. INTRODUCTION

The interaction between a pathogen and a host organism leads to a competitive relationship where both protagonists try to defeat each other. A virulent pathogen produces virulence factors enabling it to gain access to essential nutriments, to adapt to new environmental conditions and to overwhelm host defence. Specific high-affinity Fe transport mechanisms and perturbation of host cellular Fe homeostasis represent a remarkable strategy involved by bacterial pathogens to deal with the conditions imposed by the host during infection (Ratledge and Dover, 2000; Weinberg, 2000). Necessary for most forms of life, Fe is not readily available. The intrinsic properties of Fe, to be insoluble at physiological pH and to catalyse the generation of toxic free radicals, imply that living cells must use powerful mechanisms to acquire this metal in their environment and to store it in a safe form. Thus, to proliferate within host tissues, a pathogen must be armed with competitive Fe acquisition systems preventing it to be deprived of nutritional Fe. In plant-pathogen interactions, this question has been thoroughly investigated in the case of the bacterium *Erwinia chrysanthemi*, an extracellular parasite that infects a wide range of plants (Expert, 1999). This chapter focuses on the strategies developed by *E. chrysanthemi* to control its Fe homeostasis during its infectious cycle.

2. *Erwinia chrysanthemi*, A PATHOGEN OF AGRICULTURAL IMPORTANCE

Pectinolytic *Erwinia*, recently reclassified in the genus *Pectobacterium*, forms one of the two groups within enterobacteria that contain species pathogenic to plants. A typical member in this group is *E. chrysanthemi*, a pathogen that can prosper in various environments and cause disease in economically important plants, including vegetables (potato, chicory, maize, etc.) and ornamentals (African violet, carnation, orchids, etc.). Soft rot, the most visible symptom induced by this species, results from the degradation of pectin, the major component of primary cell walls. *E. chrysanthemi* attacks storage organs and fleshy plant tissue, particularly when physiologically compromised by bruising, excess of water or high temperature. This bacterium is also associated with systemic and latent infections in growing plants of many species. It is generally not endemic in soil (Perombelon and Kelman, 1980). It can overwinter in contaminated plant residues, remaining in the soil after harvest. Diseased plants act as foci for the spread of the bacteria to non-contaminated plants. Soil water (rainfall and irrigation), airborne insects and aerosols play a role in dissemination.

In vegetatively propagated crops, latent infections may have a dramatic effect on the production, because they become active under favourable conditions of soil, temperature and humidity often met in the greenhouse. Therefore, elucidating the mechanisms by which *E. chrysanthemi* exerts its virulence is a prerequisite for the development of molecular tools to diagnose and control soft rot diseases.

3. THE DISEASE PROCESS

Determining where *E. chrysanthemi* cells multiply and how they migrate in the plant is essential for further understanding the molecular events involved in the disease process. In natural infections, *E. chrysanthemi* enters the aerial parts of a host plant through wounds and natural openings. Experimental infections are performed by leaf infiltration using *E. chrysanthemi* 3937, a strain isolated from *Saintpaulia ionantha* H. Wendl. (African violet), and widely recognized by plant pathologists as a valuable model for the analysis of phytopathogenicity determinants at the molecular level. Cytological examination of leaf and petiole of *Saintpaulia* plants inoculated with strain 3937 revealed the occurrence of a symptom-less phase that may last several days, during which bacterial cells remain clustered in intercellular spaces of the cortical parenchyma or migrate intercellularly without severe injury of cellular structures. The symptomatic phase is consecutive to the production of pectinases, especially pectate lyase isozymes (PelA to PelE) that, by hydrolysing the pectic components of the middle lamella, progressively dissolve plant cell walls and enable the bacteria to multiply and to disseminate within the leaf and petiole (Murdoch *et al.*, 1999). If favourable conditions of humidity and temperature are met, a total collapse of the infected plant is observed. Bacterial cells do not migrate in the vascular tissue. During this infectious process, the bacteria encounter various environmental conditions, of which Fe availability and production of reactive oxygen species by the plant are two factors that can limit their spread.

4. SIDEROPHORE-MEDIATED IRON ACQUISITION IN *Erwinia chrysanthemi*

An analysis of the role of Fe in pathogenicity requires a characterization of the high-affinity Fe uptake systems produced by the pathogen in Fe-limited environments. *E. chrysanthemi* 3937 synthesizes two structurally unrelated siderophores, achromobactin and chrysobactin (Figure 10-1),

which are produced in a sequential manner in culture supernatants of bacterial cells grown under Fe limitation (Franza *et al.*, 2005). In low-Fe cultures, achromobactin can be detected during the mid-exponential phase of growth while chrysobactin appears much later. As a catecholate readily identifiable using the Arnow-test, chrysobactin was the first siderophore of strain 3937 to be characterized (Persmark *et al.*, 1989). Achromobactin is a hydroxycarboxylate siderophore that does not contain any catecholate or hydroxamate group (Münzinger *et al.*, 2000). This siderophore was uncovered in chrysobactin deficient mutants, which are still able to form a halo of discoloration on CAS agar medium, a universal assay for siderophore detection (Schwyn and Neilands, 1987). Chrysobactin-deficient mutants fail to grow in the presence of the ferric Fe chelator EDDHA, but the production of achromobactin enables them to thrive on a medium containing the ferrous Fe chelator 2,2' dipyridyl.

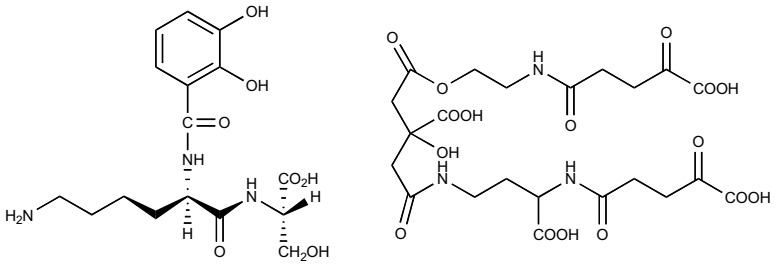

Figure 10-1. Structures of the two siderophores, chrysobactin (left) and achromobactin (right) produced by *E. chrysanthemi* strain 3937.

Structural properties of chrysobactin have been studied in detail (Persmark *et al.*, 1989). Chrysobactin, identified as *N*-[N^2-(2,3-dihydroxybenzoyl)-D-lysyl]-L-serine, belongs to a class of siderophores which are basically dihydroxybenzoic acid (DHB) derivatives of amino acids or peptides. Unlike the tricatecholate siderophore enterobactin and other hexadentate ligands, which are strong Fe binders, chrysobactin possesses only three potential coordination sites for complexing ferric Fe, two hydroxyl groups on the catechol moiety and the terminal carboxylate group of serine. Persmark and Neilands (1992) have shown that only catecholate hydroxyl groups are involved in the chelation suggesting that chrysobactin is a bidentate ligand, and thus a relatively weak ligand. However, depending on the pH and metal/ligand concentration ratio, chrysobactin was found to form ferric complexes of different stoichiometries, from 1:1 to 1:3 (Fe:chrysobactin). When ligand is four or more times in excess, there is a mixture of bis and tris complexes in solution at physiological pH. To further understand the biological function of

chrysobactin, Albrecht-Gary and coworkers have investigated the coordination properties of the different ferric complexes of this siderophore. They found that chrysobactin is a less effective ferric chelator than hexadentate siderophores, such as enterobactin or ferrioxamine B. However, chrysobactin exhibits higher pFe value than citrate or malate (pFe of chrysobactin = 17.1, vs. pFe of citrate = 14.8), which are known to be the major ferric carriers in plants, and can effectively sequester Fe from their ferric complexes (Albrecht - Gary, personal communication, September 2, 2002). Achromobactin belongs to a class of siderophores that involves carboxylate or/and hydroxy donor groups for Fe binding. Although the coordination chemistry of this ligand has not yet been investigated, it can be predicted that achromobactin is also competitive in the environment in which it is meant to function.

5. GENETICS OF CHRYSOBACTIN- AND ACHROMOBACTIN-DEPENDENT IRON TRANSPORT

A first approach based on cloning and complementation tests using *E. coli* mutants defective in enterobactin production allowed the genetic region involved in chrysobactin-mediated Fe transport to be identified. Using a collection of mutants that were unable to grow in the presence of EDDHA, it was possible to characterize the different stages required for biosynthesis of chrysobactin and the transport back to the cell of its ferric complex (Enard *et al.*, 1988). The genetic organisation of the chrysobactin system is reminiscent of that of the enterobactin region in *Escherichia coli* K12. It comprises four operons divergently transcribed from two bidirectional promoters (Figure 10-2). The *fct-cbsCEBA* operon encodes the receptor Fct and the enzymes leading to the DHB moiety in chrysobactin biosynthesis (Franza and Expert, 1991). A second operon, *cbsHF*, encodes chrysobactin synthase CbsF, which is predicted to be a nonribosomal peptide synthetase with a multimodular structure allowing the assemblage of the three components, DHB, D-lysine and L-serine (Expert *et al.*, 2004). The CbsH protein is an oligopeptidase that degrades chrysobactin in the cytosol. Removal of this enzyme by mutation results in bacterial growth inhibition caused by intracellular ferric Fe chelation (Rauscher *et al.*, 2002). Thus, this peptidase plays an important role in the control of Fe homeostasis in *E. chrysanthemi*. A third cluster of genes located upstream of the *cbsHF* operon encodes the four components of ferric chrysobactin permease (CbuBCDG) and a protein (P43) homologous to *E. coli* EntS, predicted to be involved in the excretion of chrysobactin to the external medium (Expert *et al.*, 2004).

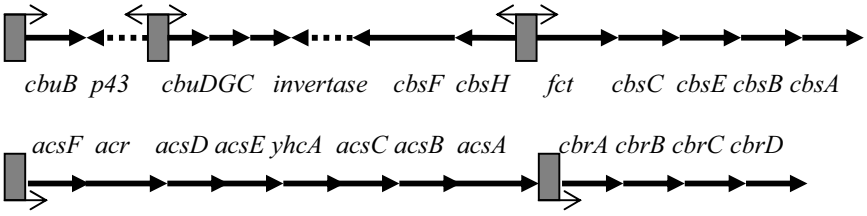

Figure 10-2. Genetic organization of the loci involved in chrysobactin (top) and achromobactin (bottom) biosynthesis (cbs and acs) and transport (fct, cbu, acr, cbr). Rectangles with arrows represent promoters and transcriptional directions. The chrysobactin degradation gene is cbsH, and the achromobactin export gene is yhcA. Arrows with dot lines depict genes, the function of which has not been determined.

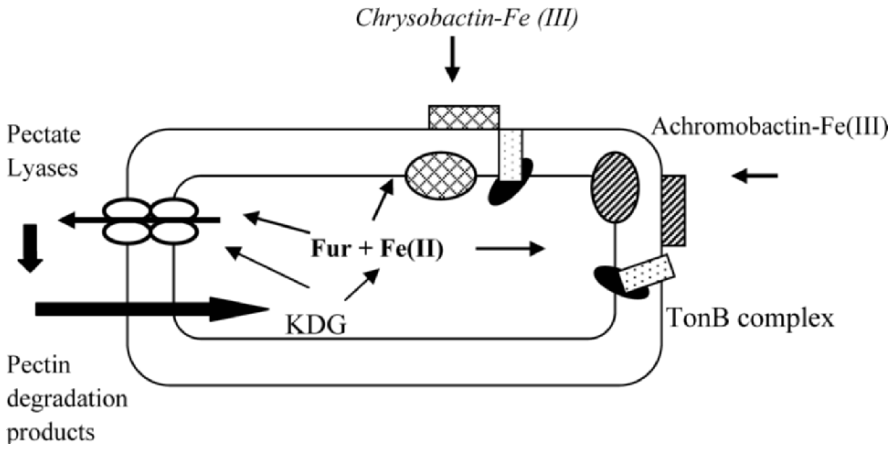

Figure 10-3. Coordinated regulation of pectinolysis and iron transport in *E. chrysanthemi* 3937. Under low-iron conditions, the Fur-mediated transcriptional repression of the genes involved in chrysobactin/achromobactin mediated iron transport and pectinolysis is relieved. In the presence of iron, intracellular accumulation of the pectin degradation product KDG turns on the transcription of the pectinase-encoding genes by inactivation of the transcriptional repressor KdgR, as well as that of the genes involved in iron acquisition by an unknown mechanism. Ferric-chrysobactin and achromobactin receptors are depicted by squared and hatched rectangles and their cognate ABC permeases by squared and hatched balls, respectively.

A second approach based on screening of mutants with altered siderophore activity on CAS-agar medium allowed to characterize the genetic region involved in achromobactin-mediated Fe transport (Mahé *et al.*, 1995) and the *tonB* gene (Enard and Expert, 2000). This gene encodes an essential component of the Ton protein machinery required for active

transport of ferric siderophores through their outer membrane receptors (Figure 10-3). The organisation of the achromobactin gene region differs from that of the chrysobactin region (Franza *et al.*, 2005). Upstream of the *cbrABCD* operon encoding the ferric achromobactin permease, a long operon comprising seven genes required for the biosynthesis (*acs*) and excretion (*yhcA*) of achromobactin, as well as the gene encoding the specific outer membrane receptor for its ferric complex (*acr*) was characterized. Three genes (*acsF acsE* and *acsB*) would be necessary for the synthesis of achromobactin precursors, diaminobutyric acid, ethanolamine and 2-oxo-glutaric acid. The *acsD*, *acsB* and *acsA* genes must encode the three components of achromobactin synthase involved in amide bonds formation. *Cbr* negative mutants that do not produce ferric achromobactin permease accumulate achromobactin in the medium and are derepressed for the production of chrysobactin.

The occurrence of two independent genetic systems for siderophore-mediated Fe transport in *E. chrysanthemi*, both regulated by Fe availability, raised the question as whether these two systems are equally involved in the pathogenicity.

6. ROLE OF CHRYSOBACTIN AND ACHROMOBACTIN IN *Erwinia chrysanthemi* PATHOGENICITY

The role of siderophore-mediated Fe uptake in the development of soft rot was determined by studying the virulence properties of simple or double mutants of strain 3937 affected in the biosynthesis of chrysobactin or/and achromobactin. Chrysobactin defective mutants produce only localized symptoms on *Saintpaulia* plants and are disadvantaged in their growth compared to a chrysobactin proficient strain (Masclaux and Expert, 1995). This decline coincided with the emergence of a necrotic border surrounding the lesion initiated by the mutant. These data indicate that chrysobactin is essential for ensuring Fe nutrition of bacteria multiplying within their host. Chrysobactin was detected in leaf intercellular fluids from plants inoculated with the wild type strain, suggesting that this compound could sequestrate the Fe present in colonized tissues and induce a plant reaction aimed at denying the bacterial cells essential Fe (Neema *et al.*, 1993). Such a competition for nutritional Fe was further investigated, using a plant-bacterial cell system that allowed accurate control of the Fe flux between both partners. Iron incorporated into plant ferritin appeared to be considerably reduced in bacteria-treated suspension soybean cells, an effect

that could be reproduced with chrysobactin or intercellular fluids from *E. chrysanthemi* inoculated leaves (Neema *et al.*, 1993).

Achromobactin deficient mutants are also affected in the virulence but are more agressive than the chrysobactin non-producers (Franza *et al.*, 2005). On the other hand, double mutants deficient in achromobactin and chrysobactin production are impaired in symptom initiation, indicating that these two siderophores act in a complementary way to satisfy the bacterial Fe needs during their passage within the plant. In this regard, the fact that achromobactin and chrysobactin have different affinity for Fe and could be produced *in planta* in a sequential manner is likely an advantage for bacterial cells. It is noteworthy that the wild type cells, but not the double mutants, can survive for several days in intercellular spaces of host tissues without multiplying substantially. In contrast, during the symptomatic phase, the number of viable counts appears to strongly increase, but the multiplication of the double mutant is ten-fold lower than that of the wild type strain. Therefore, both siderophores contribute to successful infection. Initiation of the symptomatic phase of the disease appears to be critical if there is no siderophore to be produced. The role of chrysobactin may prevail once the symptoms have started, thus indicating that Fe is not readily available, despite the degradation of tissues by pectinases.

7. CONTROL OF IRON HOMEOSTASIS AND VIRULENCE

In Gram-negative bacteria, production of a siderophore and proteins involved in uptake of its ferric complex is accurately controlled by the sensory and regulatory protein Fur. Fur protein acts as a dimer, each monomer containing a non-heme ferrous Fe site (Hantke, 2001). If the cellular Fe level becomes too low, the active Fur repressor losses Fe^{2+}, its co-repressor, and is no longer able to bind to its operator sites (Escolar *et al.*, 1999). The *E. chrysanthemi fur* gene was cloned by functional complementation of an *E. coli fur* mutant. It encodes a protein highly similar to *E. coli* Fur (Franza *et al.*, 1999). Analysis of transcriptional fusions to several genes of the chrysobactin and achromobactin systems in *fur* proficient and deficient backgrounds established that Fur is the main actor of the regulation by Fe in *E. chrysanthemi*. Monitoring the expression of a *fct-lacZ* fusion representative of the chrysobactin gene system *in planta* allowed to provide direct evidence of *in vivo* regulation of an Fe-controlled function associated with phytopathogenicity and of the low Fe availability in the plant apoplasm (Masclaux and Expert, 1995). The *E. chrysanthemi* Fur protein was purified and band shift assays demonstrated that Fur specifically binds

in vitro the regulatory regions of the genes involved in chrysobactin and achromobactin mediated Fe transport, indicating that this metalloregulation is direct (Franza *et al.*, 2002, 2005). The determination of the Fur-binding sites by DNA footprinting experiments supports the model by which the regulatory process involves direct competition between the RNA polymerase and Fur, prevailing in *E. coli* (Franza *et al.*, 2002).

Iron availability was also shown to regulate transcription of genes (*pelB* to *pelE*) encoding pectate lyases (PelB to PelE): moderate Fe limitation induces transcription of the genes *pelB*, *pelC* and *pelE*, whereas higher Fe deficiency is necessary to upregulate the *pelD* gene (Sauvage and Expert, 1994; Masclaux *et al.*, 1996). The isoenzymes PelD and PelE play a major role in virulence of strain 3937 on African violets. Inspection of the promoter region of *pelD* and *pelE* genes revealed two conserved Fur boxes located respectively 30 and 50 bp upstream from the - 35 promoter site of these two genes. In addition to their unusual position, these Fur boxes overlap the binding sites for the cAMP-CRP complex that activate transcription of *pel* genes. For these genes, the sequence protected by Fur includes a part of the CRP binding site (Franza *et al.*, 2002). Thus, in this case, Fur should act as an antiactivator of transcription by blocking the action of CRP.

The molecular data reported above indicate that low Fe availability is a triggering signal for coordinated expression of the genes encoding pectate lyases and those involved in chrysobactin/achromobactin mediated Fe uptake. Pectolyases provide bacteria with carbon sources derived from the degraded pectin. Pectin catabolism leads to the formation of 2-keto-3-deoxygluconate (KDG) (Figure 10-3), which is the main inducer of this metabolic pathway: transcription of the genes involved in pectinolysis is repressed by a common regulator, KdgR. In the presence of pectic inducers like KDG and Fe, the repressor KdgR is inactivated and Fur is active and can thus prevent the activation of the *pelD* and *pelE* genes by CRP. In addition, the negative metalloregulation by Fur of Fe transport genes was found to be partially relieved during pectinolysis, regardless of the Fe level, indicating that additional effectors are involved in this regulatory circuit to make it fully efficient. An insertional mutagenesis of the chromosome was carried out in order to find mutations conferring increased expression of both *pelD* and *fct* genes (Franza *et al.*, 2002). One insertion giving rise to this phenotype appeared to inactivate the expression of the *kdgK* gene encoding the KDG kinase. The KDG phosphorylation is the last step of the pectin or 3-galacturonate catabolic pathway, before this compound is converted into phosphoglyceraldehyde and pyruvate. A mutation in the *kdgK* gene, which blocks this metabolic conversion, leads to the intracellular accumulation of KDG and de-represses genes involved in pectinolysis. The effect of the *kdgK*

mutation on de-repression of the *fct* gene in the presence of Fe was intriguing. This effect can be reproduced by addition of KDG in the culture medium and is not mediated by the regulator KdgR. These data indicate that intracellular accumulation of KDG modulates not only the expression of the pectate lyases encoding genes but also that of Fe transport genes. One interpretation of these results is that this compound acts directly on the Fur regulatory activity. It seems thus that the two pathogenicity determinants, Fe acquisition and pectinolysis, are regulated in a coordinate manner by responding to common signals, Fe availability and pectin (Figure 10-3). This metabolic coupling should confer an important advantage for the bacteria during pathogenesis.

8. CONTROL OF IRON HOMEOSTASIS AND DEFENSE AGAINST OXIDATIVE STRESS

In response to an attack by microbial pathogens, plant cells produce active oxygen species (AOS), which include superoxide anion (O^{2-}), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\cdot}$). This production of AOS is termed oxidative burst. Oxidative burst is an important component of the plant host defense system (Lamb and Dixon, 1997). Furthermore, these reactive oxygen species are involved in the induction of plant immunity (Levine *et al.*, 1994; Alvarez *et al.*, 1998). In disease situations, the role of AOS has been investigated after infection by *E. chrysanthemi* (Santos *et al.*, 2001). Generation of superoxide anion by African violet inoculated with *E. chrysanthemi* was demonstrated with nitroblue tetrazolium as an indicator. Besides cell-wall degrading enzymes and Fe acquisition systems that proved to be necessary for symptom spreading, a manganese superoxide dismutase (Santos *et al.*, 2001), a peptide methionine sulfoxide reductase, that repairs oxidized proteins (El Hassouni *et al.*, 1999) and a [Fe-S] cluster assembly/repair machinery (Suf) (Nachin *et al.*, 2001) confer on the bacterium the ability to survive *in planta* and to cope with damage caused by oxidative stress during infection. This Suf machinery is encoded by an operon of six genes, which is negatively controlled by Fur. In particular, this machinery is active in bacterial cells exposed to oxidative conditions or to severe Fe limitation. Intracellular Fe acquisition *via* chrysobactin is impaired in *suf* negative mutants, indicating that the Suf machinery could participate to the formation of an [Fe-S] cluster containing protein required for Fe liberation from ferric chrysobactin (Nachin *et al.*, 2003). In this respect, the Suf system appears to be a key component in bacterial Fe homeostasis during infection.

9. CONCLUSION AND PROSPECTS

Studying the role of siderophores in the pathogenicity of *E. chrysanthemi* 3937 has contributed to an appreciation of the regulatory mechanisms involved in the expression of virulence by plant-pathogenic bacteria. The ability of this strain to infect a large number of hosts is probably related to the performance of its pectolytic equipment. A rapid destruction of plant tissues must allow the bacteria not only to gain access to plant nutrients but also to overcome the host defences. The existence of a fine-tuning mechanism such as Fe sensing to ensure a balanced production of pectate lyases during the infection process is probably a selective advantage for strain 3937. To control its Fe homeostasis, this bacterium produces two different siderophores, achromobactin and chrysobactin, whose contribution are probably modulated by the Fe fluctuations encountered during the infection process. Thus, the individual role of these two siderophores *in planta* has to be explored. It was also shown that Fe sensing by bacterial cells during infection is an efficient way to respond to the oxidative burst elicited in the plant. This indicates that these bacteria must accurately control their intracellular Fe utilization and cope with changes in redox conditions, by the use of mechanisms that need to be elucidated. Bacterial ferritins and ferritin-like proteins are good candidates for being involved in these processes. Another important question is whether plants control and modify their Fe storage during infection, possibly by developing reactions similar to the Fe-withholding response induced by animal and human pathogens. As *E. chrysanthemi* 3937 can induce systemic symptoms in the model plant *Arabidopsis thaliana*, this should help to unravel plant Fe mobilization reactions that may occur during pathogenesis. This point may be of importance if infection occurs on plants growing under Fe stressing conditions. Perturbations of nutritional Fe compromise plant health and crop production and elucidating mechanisms involved in exchanging and withholding Fe during plant microbe-interactions should help to develop integrative strategies for controlling plant diseases.

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Chapter 11

IRON STRESS RESPONSES IN ROOTS OF STRATEGY I PLANTS

WOLFGANG SCHMIDT
Biological Science and Bioengineering Program, Faculty of Engineering and Natural Science, Sabanci University, 34956 Istanbul, Turkey. E-mail: wolfgang.schmidt@botany.de

Abstract: In concert with phosphorus and nitrogen, iron belongs to the nutrients that most commonly restrict growth of plants. Being rapidly oxidized and immobilized, the more soluble ferrous species is rarely available in sufficient amounts; instead, ferric iron is mainly present in oxi-hydrates that cannot be taken up by plants. Plants have evolved a syndrome of responses to balance iron levels within cells, comprising of sensing the levels of available iron in leaves and roots, mobilization of iron pools in the immediate vicinity of root epidermal cells, and uptake and distribution within the plant. This system appears to be conserved among plants. When iron sources are limited, in dicots and non-graminaceous monocots a reduction-based iron uptake mechanism is induced which is not iron-regulated in grasses. The major differences between these two groups of plants seems to be related i) to the control of the iron reduction/iron uptake step in iron acquisition, and ii) in the evolution of the formation of iron-avid phytosiderophores in grasses, which allows uptake of iron without prior reduction.

Key words: intracellular transport; ion homeostasis; iron acquisition; local and systemic signaling; morphologic adaptations

1. INTRODUCTION

The evolution of oxygenic photosynthesis during the Archaean led to a dramatic increase in the ambient oxygen level which was initially absorbed by ferrous iron, oxidizing it to Fe(III) oxide. The extremely poor solubility of the oxidized, ferric form led to bioavailability problems, which forced living organisms to develop mechanisms for obtaining iron from the environment. Generally, these mechanisms involve the solubilization of extracellular iron,

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either by reduction or by chelation, followed by internalization via specific transporters. Fungi, grasses and most bacteria synthesize and secrete siderophores, which are low molecular weight compounds that specifically bind ferric iron with high affinity, the siderophore-iron complex being captured by specific cellular transport systems. Some bacteria, yeast and most higher plants use cell surface reduction to convert ferric iron to soluble ferrous species. Among plants, this mechanism has been referred to as Strategy I, in contrast to the siderophore-based iron acquisition system of grasses (Römheld and Marschner, 1986). The Strategy I iron stress syndrome comprises physiological, developmental and metabolic reactions, adapting the plant to changing levels of available iron resources. Transcriptome analysis has revealed that a diverse array of genes is differentially expressed upon iron deficiency, indicating a broad range of processes that are either activated or down-regulated when iron availability is limited (Thimm *et al.*, 2001). Since iron is potentially toxic, iron acquisition and storage systems are regulated in response to cellular needs and to the spatial and temporal availability of iron. An overview of genes involved in iron uptake and homeostasis is given in Table 11-1.

Iron shortage reduces the yield of crop plants and causes a decrease in nutritional value of edible plant parts. Therefore, iron homeostasis of plants is a highly active research area, which is reflected by numerous publications in the last few years, the outcome of which has been summarized in several recent reviews (Curie and Briat, 2003; Schmidt 2003; Hell and Stephan, 2003; Bauer and Bereczky, 2003). This chapter focuses on recent research advances concerning the components and regulation of reduction-based iron uptake in higher plants. Although it is attempted to provide a general overview, a special focus is given on more recent breakthroughs in iron research. Other chapters dealing with Strategy I responses in this book are by Bauer and Hell (Chapter 13), Hall and Guerinot (Chapter 15), Rogers (Chapter 16), Zocchi (Chapter 18) and Reinhardt *et al.* (Chapter 19).

2. ELECTROGENIC CHELATE CRACKING: IRON REDUCTION

Reduction of ferric chelates at the plasma membrane is an obligatory step prior to uptake in Strategy I plants (Chaney *et al.*, 1972). Increased Fe(III) reductase activity in response to iron starvation has been observed in all Strategy I plants investigated so far and can thus be considered as a core feature of this strategy. All characterized ferric reductase genes (*AtFRO2*, *LeFRO1* and *PsFRO1*) encode polypeptides of 712 to 725 amino acids with molecular masses of 80.5 to 81.5 kDa. The proteins contain binding sites for

Table 11-1. Genes with a proven or putative function in iron homeostasis in strategy I plants. N.r.: not reported.

Gene	Putative function	Response to iron deficiency	Highest expression	Subcellular localization	References
AtFRO2	Iron mobilization	induced	Epidermis		Robinson *et al.*, 1999 ; Connolly *et al.*, 2003
AtFRO3	Iron mobilization	induced	n.r.	n.r.	Robinson *et al.*, 1999; Wintz *et al.*, 2003; Bauer *et al.*, 2004
PsFRO1	Iron mobilization	induced	Epidermis	n.r.	Waters *et al.*, 2002
LeFRO1	Iron mobilization	induced	n.r.	Plasma membrane	Li *et al.*, 2004
AtIRT1	Iron uptake	induced	Epidermis	Plasma membrane	Eide *et al.*, 1996; Vert *et al.*, 2002
AtIRT2	Iron sequestration	induced	Epidermis	Intracellular membranes	Vert *et al.*, 2001; 2004
PsRIT1	Iron uptake	induced	n.r.	n.r.	Cohen *et al.*, 2004
LeIRT1	Iron uptake	induced	Epidermis	Plasma membrane	Eckhardt *et al.*, 2001; Schikora *et al.*, unpublished
LeIRT2	Unclear	not affected	Epidermis and vascular parenchyma	n.r.	Eckhardt *et al.*, 2001
AtYSL2	Lateral movement of metals in the vasculature	down-regulated	Different cell types	Plasma membrane	DiDonato *et al.*, 2004
AtOPT3	Metal transport	induced	Vascular tissue	n.r.	Stacey *et al.*, 2002; Wintz *et al.*, 2003
AtCCC1	Mobilization of cytosolic metals into vacuoles	n.r.	n.r.	Vacuole	Kim *et al.*, 2004
AtNRAMP3	Mobilization of vacuolar metal pools	induced	Vascular tissue	Vacuole	Thomine *et al.*, 2003
AtNRAMP4	Unclear	induced	Vascular tissue	Vacuole	Thomine *et al.*, 2004,
LeNRAMP1	Acquisition or redistribution	induced	Epidermis, cortex, vascular tissue	Intracellular vesicles	Bereczky *et al.*, 2003
LeNRAMP3	Unclear	slightly induced	n.r.	diffus	Bereczky *et al.*, 2003
GmDMT1	Nodule Fe homeostasis	n.r.	Nodules	PMB	Kaiser *et al.*, 2003
AtAHA1,2,4,7	Iron mobilization?	induced	Epidermis	Plasma membrane	Santi *et al.*, unpublished
CsHA1	Iron mobilization?	induced	n.r.	n.r.	Santi *et al.*, unpublished
AtFRD3	Xylem loading	induced	Pericycle, vascular cylinder	Plasma membrane (?)	Rogers and Guerinot 2002; Green and Rogers, 2004
LeFER	Regulation Fe homeostasis	slightly down-regulated	Epidermis, cortex, vascular tissue	Nuclei	Ling *et al.*, 2003; Brumbarova and Bauer, 2005
AtFIT1/AtFRU	Regulation Fe homeostasis	induced	Epidermis		Colangelo and Guerinot, 2004; Jakoby *et al.*, 2004
AtFER1	Iron storage	down-regulated	Root endodermis, near leaf	n.r.	Tarantino *et al.*, 2003

FAD and NADPH, consistent with a function in transferring electrons from cytosolic pyridine nucleotides to ferric chelates on the opposite site of the membrane (Robinson *et al.*, 1999; Waters *et al.*, 2002; Li *et al.*, 2004). The ferric chelate reductase of *Arabidopsis* is a gene family consisting of eight members. Expression of *AtFRO2* in roots is induced by iron deficiency and was shown to complement *frd1-1*, a loss-of-function mutation in *FRO2* that does not induce Fe(III) chelate reductase activity under iron-deficient growth conditions (Yi and Guerinot, 1996; Robinson *et al.*, 1999). The function of other *FRO* genes in *Arabidopsis* has not been clarified until now. Ferric iron chelates delivered via the xylem are reduced before the divalent ion is taken up by leaf cells (Abadía *et al.*, 2002; Brüggemann *et al.*, 1993). Expression of *AtFRO3* was found to be iron-regulated in both leaves and roots, indicating a possible function of FRO3 in leaf iron uptake (Wintz *et al.*, 2003).

In contrast to *Arabidopsis*, in which *FRO2* expression is restricted to the roots, the reductase gene *LeFRO1* in tomato is expressed in both roots and leaves. In leaves, its expression is not affected by iron starvation and is also present in the *fer* mutant, which shows no detectable levels of *LeFRO1* transcripts in the roots, suggesting that regulation of ferric reduction in leaves differs from that of roots (Li *et al.*, 2004). A separate regulation of reductase gene expression in roots and leaves was also reported for pea roots. Reductase activity and mRNA levels of *PsFRO1* are constitutively up-regulated in roots of *brz* and *dgl*, mutants defective in iron uptake regulation, but gene expression was found to be iron-responsive in leaves of the mutants and their wild-type (Waters *et al.*, 2002). Thus, in leaves, *FRO* genes can be either not expressed, iron-responsive, or non iron-responsive, suggesting variations in the importance of a reductive step in leaf iron uptake.

3. CROSSING BORDERS: IRON UPTAKE

The major route for the entry of iron into root cells is IRT1, a protein belonging to the ZIP (Zn-Fe-regulated transporter) family of metal transporters, first identified in *Arabidopsis* (Eide *et al.*, 1996). The knockout mutant of the *IRT1* gene shows chlorosis symptoms similar to those induced by iron deficiency, reduced iron accumulation and reduced growth, which can be restored after additional iron supply (Henriques *et al.*, 2002; Varotto *et al.*, 2002; Vert *et al.*, 2002). Homologues of IRT1 have been cloned from tomato (*LeIRT1*) (Eckhardt *et al.*, 2001) and pea (*RIT1*) (Cohen *et al.*, 2004). A K_m for ferrous iron between 54 to 93 nM has been reported for RIT1 which is consistent with the Fe^{2+} activity that has been estimated to occur in soil solutions (Cohen *et al.*, 2004). Although IRT proteins are relatively

unspecific for iron and transport other metals such as Zn and Cd as well, they have a higher affinity for Fe (Cohen *et al.*, 2004). Thus, at low external metal concentrations iron may be taken up preferentially over other metals.

Expression of IRT is induced by iron deficiency in the epidermis of roots (Vert *et al.*, 2002). *AtIRT1* is regulated both at the level of transcription and protein accumulation. In *35S-IRT1* transgenic plants, the IRT1 protein was only present in iron-deficient plants, whereas *IRT1* mRNA was expressed constitutively. At sufficient iron supply to the roots IRT1 protein may be ubiquitinated and subsequently degraded (Connolly *et al.*, 2002). This dual regulation of IRT1 activity provides a mechanism that allows a fast turn-off of iron uptake at high external levels which may otherwise lead to potentially toxic levels when uptake systems are up-regulated. A similar regulation has been demonstrated for *AtFRO2*, indicating that regulation of reduction activity is closely coupled to that of iron uptake, both at the transcriptional and post-transcriptional level (Connolly *et al.*, 2003). Split-root experiments have shown that *IRT1* and *FRO2* mRNA levels can be stabilized by a remote signal from the shoot even at adequate external iron levels, when the overall iron status of the plant is low. Expression of both genes is down-regulated during the night, a response which is superimposed over the prevalent nutritional status signals (Vert *et al.*, 2003a).

4. PUMPING PROTONS: RHIZOSPHERE ACIDIFICATION

The solubility of iron depends largely on proton activity, being decreased up to 1,000 fold for each unit increase in pH. Thus, weakening of the Fe-O bonds by acidification of the rhizosphere is an efficient means to increase the availability of iron. Net proton excretion has been described for many species (e.g. tomato, cucumber and sunflower) and has been attributed to an increased activity of a P-type H^+-ATPase at the plasma membrane (Römheld *et al.*, 1984; Dell`Orto *et al.*, 2000; Schmidt *et al.*, 2003). In roots of tomato, H^+-ATPase protein density was specifically enriched in transfer cells formed upon Fe-deficiency in the rhizodermis, consistent with a function of this cell type in proton efflux (Schmidt *et al.*, 2003). Recently, accumulation of a specific ATPase isoform, *CsHA1,* in response to iron deficiency was observed in roots of cucumber. A housekeeping isoform, *CsHA2*, expressed in both roots and leaves, was not affected by the iron nutritional status (Santi *et al.*, 2005).

In *Arabidopsis*, the family of P-type ATPases consists of 12 members, the function of which has not been determined in most of the cases until now (Palmgren, 2001). *AtAHA2* is preferentially expressed in root hairs, and may

function in the uptake of nutrients (Sussmann, 1994). At least four ATPase isoforms showed increased mRNA steady state levels upon iron deficiency, among which *AtAHA2* exhibits highest transcript abundance (Santi *et al.*, in preparation). This supports earlier observations that GUS activity driven by the *AHA2* promoter is increased in the epidermis of Fe-deficient *Arabidopsis* roots (Fox and Guerinot, 1998).

In contrast to the ubiquitous increase in ferric chelate reduction, acidification of the rhizosphere is not evident in all Strategy I species, and, in those species generally showing this response, it depends on several factors such as the cation/anion uptake balance, root exudations, and on the form of nitrogen nutrition. Besides increasing the solubility of iron, other functions of enhanced proton secretion can be considered. In tomato, the expression of *LeIRT1* and *LeFRO1* is increased at low external pH (H.Q. Ling, pers. comm.), suggesting a regulatory role of P-type ATPases. Moreover, substrate affinity of the ferric chelate reductase increases with decreasing pH, which may be attributed to a neutralization of negative surface charges thus preventing the repulsion of negatively charged Fe chelates from the site of reduction (Schmidt and Janiesch, 1991; Cohen *et al.*, 1997).

Although excretion of reducing or acidifying compounds has been generally considered as being of minor importance for the acquisition of iron, in some cases such as the formation of cluster roots, the exudation of organic acids like citrate can reach enormous levels and may have a high impact in iron availability. Reduction of H^+-ATPase activity by antisense constructs has been shown to decrease citrate efflux in a carrot mutant cell line with enhanced citrate excretion, suggesting a requirement for H^+-ATPase activity in this process (Ohno *et al.*, 2004).

5. A COMPLEX(ED) STORY: IRON POOLS IN PLANT CELLS

After uptake, excess iron has to be complexed or sequestered within the cell to avoid toxicity due to Fe(II)-mediated generation of free radicals via Fenton chemistry. In both Strategy I and Strategy II plants, intracellular delivery of iron and trafficking within the plant is dependent on nicotianamine (NA), which is believed to protect the cell from oxidative damage by forming stable complexes with Fe(II) and supporting its cellular distribution (von Wirén *et al.*, 1999). NA is a polyamine formed by the the trimerization of three molecules S-adenosylmethionine to one molecule of NA via nicotianamine synthase (NAS) (Herbik *et al.*, 1999; Higuchi *et al.*, 1999), found in all multicellular plants investigated to date (Noma and

Noguchi, 1976; Rudolph *et al.*, 1985). The NA-less tomato mutant *chloronerva* shows severe symptoms of chlorosis despite having high iron levels in the symplasm, supporting a function for NA in intra- and/or intercellular distribution of iron (Stephan and Scholz, 1993; Becker *et al.*, 1995). Overexpression of the *Arabidopsis* NAS gene *AtNAS1* led to a better growth and increased iron contents at limited iron supply (Douchkov *et al.*, 2002). The function of NA is not restricted to iron homeostasis. NA is a strong chelator for a variety of transition metals and was proven to be involved in the long distance transport of copper (Pich and Scholz, 1996) and in Zn homeostasis (Weber *et al.*, 2004). In Strategy II species NA is a precursor of phytosiderophores (see Chapter 20 by Kobayashi, Nishizawa and Mori).

Nitric oxide (NO) has been suggested to improve the availability of iron in plants by the formation of dinitrosyl-iron complexes (Graziano *et al.*, 2002). Application of NO reverted the phenotype of mutants defective in iron acquisition of both iron uptake strategies, which is consistent with a function of NO in the delivery of iron. In plants, NO can be produced either *via* nitrate reductase, by converting nitrite to NO, or by the oxidation of arginine to citrulline by nitric oxide synthase (NOS) (Neill *et al.*, 2003). A plant nitric oxide synthase has recently been identified in *Arabidopsis*, yet with no sequence similarities to any of the mammalian isoforms (Guo *et al.*, 2003). An *AtNOS1* insertional mutant, exhibiting reduced NOS activity, was impaired in leaf greening, shoot growth, and fertility. A further way of NO production in plants occurs *via* the non-enzymatic reduction of apoplastic nitrite, a process which requires a low pH (Bethke *et al.*, 2004). Assuming a substantial role of NO in iron homeostasis, increasing NO production via acidification of the apoplast may indicate a further possible function of iron deficiency-induced H^+-ATPases activity in plant cells. The use of methodologies that correct for the removal of free NO by superoxide production revealed that basal production of NO may be higher than previously estimated (Vanin *et al.*, 2004). However, a critical role of NO in iron delivery remains to be established. The possible involvement of NO in signaling is discussed below.

Information on the transport of iron into organelles is scarce. *STARIK* encodes the mitochondrial ABC transporter STA1, a functional ortholog of the yeast mitochondrial Atm1p transporter. Loss-of-function mutants of the *STARIK* gene are chlorotic, probably due to a misbalance of intracellular iron homeostasis (Kushnir *et al.*, 2001). Atm1p is likely to be involved in the export of Fe/clusters from the mitochondrial matrix into the cytosol, a similar function may be assumed for STA1. *AtSLB*, a gene with similarities to bacterial *NifS* genes which are required for the assembly of Fe/S clusters, may play a role in iron transport. AtSLB is predicted to be localized in

chloroplasts and is down-regulated upon iron deficiency, which is in agreement with a function in iron import into chloroplasts (Wintz *et al.*, 2003). A further candidate acting for iron import into plastids is NRAMP1, for which a possible localization in plastids has been predicted (Curie *et al.*, 2000). The importance of mitochondrial Fe metabolism for cellular Fe homeostasis has been indicated by the isolation of *AtISU1*, which is proposed to assist the assembly of Fe-S clusters in mitochondria (Tone *et al.*, 2004).

6. SEND AND DELIVERED: INTER- AND INTRACELLULAR TRANSPORT OF IRON

Immunochemical localization of NA in plants fed with high iron concentrations revealed that under these conditions NA, and presumably the Fe(II)NA complex, is accumulating in the vacuole (Pich *et al.*, 2001). A similar pattern was observed in iron over-accumulating mutants, supporting a function of NA in vacuolar iron sequestration. Two members of the oligopeptide transporter (OPT) family, *AtOPT2* and *AtOPT3* are highly induced by iron deficiency, and thus candidates for transport of chelated iron (Wintz *et al.*, 2003). Under control conditions, *AtOPT3* is mainly expressed in the vascular tissue of both roots and leaves (Stacey *et al.*, 2002), but a subcellular localization has not been reported so far. A family of metal transporters closely related to that of the OPT and homolog to the maize phytosiderophore transporter ZmYS1 has been identified in *Arabidopsis* (Curie *et al.*, 2001). Yellow Stripe-like (YSL) proteins lack sequences that target proteins to intracellular membranes and may localize to the plasma membrane. However, a role for YSL transporters in intracellular iron transport cannot be excluded at present. One member of this family has been characterized in detail. AtYSL2 transports both Fe(II) and copper when these metals are bound to NA and it is expressed mainly in cells of the vasculature (DiDonato *et al.*, 2004). *AtYSL2* is negatively regulated by iron deficiency, i.e. transcript abundance was found to be reduced at low iron availability. This pattern of regulation suggests that AtYSL2 contributes to the uptake of iron that has arrived from xylem transport (DiDonato *et al.*, 2004). NA is required for transporting iron from veins to the interveinal area, as can be deduced from the phenotype of the NA-less tomato mutant *chloronerv*a, which shows green veins and chlorotic intercostal areas. Similarly, transgenic tobacco that constitutively express the enzyme nicotinanamine aminotransferase (NAAT) from barley, which, in Strategy II plants, catalyzes the amino group transfer of NA to form phytosiderophores, displays interveinal chlorosis due to a lack of free NA pools (Takahashi

et al., 2003). Transport of iron complexed by NA *via* a member of the YSL family may represent the main route for the entry of iron in interveinal tissue cells. A model outlining the transport processes in root cells of Strategy I plants is shown in Figure 11-1.

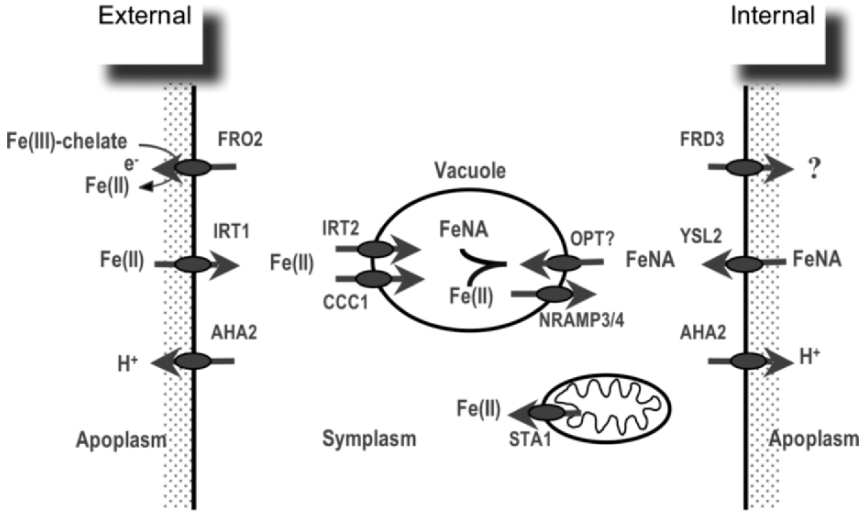

Figure 11-1. Mechanisms of iron uptake and intracellular sequestration of the Strategy I plant *Arabidopsis thaliana.* The left part of the figure is representative for the uptake of iron from the rhizosphere by peripheral cells. The right side of the figure presents a cell near or in the vasculature; the presence of transporters between the two cell types is, however not mutually exclusive. Ferric chelates are reduced by FRO2 and the released ferrous ion is subsequently transported into the cell by IRT1. Protons released into the rhizosphere by AHA2 helps to mobilize sparingly soluble iron pools and may contribute to the regulation of iron uptake. Within the cell, iron is transported into the vacuole either via IRT2 or CCC1. From the apoplasm of internal cells, iron is internalized as Fe(II)NA via YSL2. FRD3 is involved in the xylem loading or unloading, probably by exporting a chelator necessary for the long distance transport of iron. Transport processes are facilitated by AHA2. Fe(II)NA is translocated into the vacuole by an unknown transporter, possibly belonging to the OPT family. In the vacuole iron is stored as a complex with NA. Export occurs via NRAMP3 and NRAMP4 in ionic form. STA1 may be involved in the maturation of Fe/S clusters by transporting iron out of mitochondria.

A further candidate for transporting iron into the vacuole is IRT2, a close homolog to IRT1. IRT2 was found to be co-regulated with IRT1 in *Arabidopsis*, but *irt2* mutants did not display chlorosis symptoms and *IRT2* did not rescue the *irt1* mutant (Vert *et al.*, 2001; Varotto *et al.*, 2002). *AtIRT2* is expressed in intracellular membranes predominantly in the epidermis, probably representing an overflow mechanism that sequesters iron into the vacuole (Vert *et al.*, 2003b). In tomato, *LeIRT2* is expressed in

the epidermis and in the vascular parenchyma and was found to be independent of the iron nutritional status of the plant (Eckhardt *et al.*, 2001; Bereczky *et al.*, 2003). A function of LeIRT2 has not been elucidated yet.

In *Arabidopsis* and potentially in other plants, an additional way for the entry of iron (and other metals) into the vacuole may be provided via the homolog of the yeast *CCC1* gene (Kim *et al.*, 2004). AtCCC1 shows 62% similarity to yeast CCC1, which is suggested to be involved in the transport of manganese and iron into the vacuole (Li *et al.*, 2001). AtCCC1-GFP fusion localizes to the vacuolar membrane, supporting a role of CCC1 in intracellular iron trafficking.

NRAMPs (natural resistance-associated macrophage proteins) are a class of integral membrane proteins found in bacteria, fungi, plants, and animals that are capable of transporting a broad range of metals (Cellier *et al.*, 1995). The NRAMP family in *Arabidopsis* comprises six members. Two members of this family, *AtNRAMP3* and *AtNRAMP4* are induced by iron starvation and complement an iron uptake-defective mutant in yeast (Thomine *et al.*, 2000). Both proteins localize to the vacuolar membrane (Thomine *et al.*, 2003; 2004). Overexpression of *AtNRAMP3* causes decreased expression of *AtFRO2* and *AtIRT1*, which can be explained by a function of AtNRAMP3 in mobilizing vacuolar metal pools to the cytoplasm. NRAMP3 is restricted to the vascular tissue of roots and leaves, indicative of a function in long distance transport of metal ions. In tomato, release of metals from intracellular vesicles may me mediated by LeNRAMP1 (Bereczky *et al.*, 2003).

7. LONG AND WINDING ROADS: INTER-ORGAN TRANSPORT OF IRON

Several plant genes have been identified that may be involved in long-distance transport, the function of which and the underlying mechanisms are discussed elsewhere in this book (see Chapters 13 and 16). One of the best characterized enzymes involved in iron trafficking is FRD3, a member of the MATE family which is likely to play a role in shoot iron localization (Rogers and Guerinot, 2002; Green and Rogers, 2004). In *frd3* mutants iron is transported to the shoots in a form that cannot be taken up by leaf cells. Most likely, FRD3 transports not iron but a molecule that is necessary for xylem unloading of iron. A FRD3-like gene (*OsFRD1*) is expressed in companion cells of rice roots (Inoue *et al.*, 2004).

8. FORM FOLLOWS FUNCTION: SHAPE AND FATE OF EPIDERMAL CELLS

Since iron solubility is limited in most soils, an increase in the root's absorptive area, either achieved by the formation of transfer cells bearing wall protuberances at the peripheral cell walls or by the development of extra root hairs, appears to be advantageous for growth and competitiveness when iron availability is limited. Mutants producing more root hairs have higher ferric reductase activity, supporting this assumption (Schmidt *et al.*, 2000b).

In *Arabidopsis*, specification of epidermal cells in both roots and leaves is controlled by a common suite of putative transcription factors, including the homeodomaine leucine zipper protein GLABRA2 (GL2), the MYB-type transcription factors GLABRA1 (GL1) and WEREWOLF (WER), the bHLH protein GLABRA3 (GL3), and a WD40 repeat containing protein named TRANSPARENT TESTA GLABRA (TTG). Other genes that contribute to the determination of cell fate are *TRIPTYCHON* (*TRY*) and *CAPRICE* (*CPC*), encoding single-repeat MYBs (Larkin *et al.*, 2003; Bernhardt *et al.*, 2003; Wada *et al.*, 2002). In roots, cell fate determination is further affected by a positional signal that determines the patterning of hair and non-hair cells. Hairs are only formed in positions in which the underlying cortical cells form periclinal walls. GL2 acts as a repressor of the hair cell fate and is mainly expressed in cells in non-hair cell positions. The formation of GL2 is controlled by a complex consisting of WER, TTG, and GL3. This complex controls also the formation of CPC. High CPC concentrations in cells in the hair position repress both *GL2* and *WER*, thus promoting the hair cell fate (Schiefelbein, 2000; see Birnbaum and Benfey, 2004 for a recent review). Interestingly, these transcription factors are playing opposite roles in the formation of single cell trichomes and root hairs; in leaves TTG, GL1 and GL2 promote the formation of trichomes, whereas in roots TTG, WER and GL2 are positive regulators of the non-hair cell fate. TRY and CPC inhibit trichome specification and differentiation of non-hair cells (Schellmann *et al.*, 2002).

A shortage in both iron and phosphate can affect the pattern of epidermal cells, leading to an increase in density and, in the case of P deficiency, length of the hairs (Ma *et al.*, 2001; Schmidt and Schikora, 2001). Root hairs formed under conditions of iron deficiency differ from those formed under normal conditions and from hairs that developed in low phosphate media. In iron-deficient *Arabidopsis* plants, an increase in root surface area is mainly achieved by the formation of bifurcated hairs, the number of which does not differ significantly from that observed under control conditions (Müller and Schmidt, 2004). Thus, in roots of iron-deficient plants two different types of

hairs develop, i.e. unbranched hairs with characteristics similar to those formed under control conditions, and bifurcated hairs forming two tips. Analysis of mutants harboring defects in cell specification and in different stages of root hair development revealed that each type of hair is dependent on a specific set of genes. In *ttg1* and *gl2* mutants, root hair formation is promoted whereas trichome formation is abolished (Schellmann *et al.*, 2002). In contrast to hairs formed under control or –P conditions and to normal (non-branched) hairs of iron-deficient plants, mutations in *TTG* and *GL2* decreases the number bifurcated hairs. Thus, branched hairs develop in a background which promotes the development of trichomes in the leaf epidermis. The upstream components involved in sensing iron and the downstream targets of the cell specification genes have not been identified so far.

9. LOST IN TRANSLATION? SENSING AND TRANSDUCTION OF IRON DEFICIENCY SIGNALS

9.1 Iron Sensing

A sensor for iron in plant cells has not yet been identified. In animals, iron regulatory proteins (IRPs) regulate the stability of mRNAs containing iron responsive elements (IRE) located in the untranslated regions of iron-regulated transcripts (Eisenstein, 2000). By means of an Fe/S-cluster-dependent switch in response to nitric oxide, cytosolic aconitase can be converted from an enzyme that converts citrate into isocitrate to an RNA binding posttranscriptional regulator. Plant aconitases have significant homology to mammalian IRPs (Peyret *et al.*, 1995), and NO was shown to inhibit tobacco aconitase (Navarre *et al.*, 2000), supporting the possibility that similar systems for sensing iron may exist in plants. However, a functional conversion of a plant aconitase to an active IRP has not yet been demonstrated.

Profiling NO-responsive gene expression using the whole genome ATH1 microarray revealed that about 2% of the genes in *Arabidopsis* are differentially expressed after NO application (Parani *et al.*, 2004). From these genes about 10% were transcription factors, suggesting that NO is a player in multiple signal transduction pathways. In analogy to the IRP/IRE system in animals, regulation of ferritin via an NO-mediated pathway has recently been demonstrated by Murgia *et al.* (2002). In plants, repression of ferritin synthesis under low iron supply occurs at the level of transcription and thus differs from the regulation in animals based on stability and

translatability of mRNA. Interestingly, among the four ferritin genes present in *Arabidopsis*, *AtFer1*, *AtFer3* and *AtFer4* were found to be responsive to both iron (Petit *et al.*, 2001) and NO (Parani *et al.*, 2004) in the same order of strength, whereas *AtFer2* was not affected by either treatment.

In *Arabidopsis*, NO bioactivity is affected by the nonsymbiotic hemoglobin AHb1, and scavenging of NO by hemoglobin was suggested to take part in the modulation of NO-mediated signal systems (Perazzolli *et al.*, 2004). Interestingly, the tomato hemoglobin gene *SOLly GLB1* is up-regulated in response to deficiencies in a variety of nutrients, including iron (Wang *et al.*, 2003). This opens the possibility that the bioactivity of NO is affected by nutrient starvation situations and is controlled via non-symbiotic hemoglobin. However, such a response mechanism is highly speculative and needs further experimental support.

A sensor for iron might be located either in the plasma membrane, sensing the external iron concentration, or in the cytoplasm, measuring the intracellular iron level. Since IRT1 knock-out mutants exhibit a higher ferric chelate reductase activity and more root hairs relative to the wild-type at sufficient iron conditions, a putative sensor may be located inside the cell (Vert *et al.*, 2003a). NA has been assumed to be involved in iron sensing by mediating its binding to trans-acting factors, but downstream targets of this (hypothetical) mechanism have not been yet identified. Binding of other metals may interfere with NA-mediated iron perception. In support of this view, high zinc levels induced expression of iron-related genes in *Arabidopsis* (*IRT2*) (Wintz *et al.*, 2003) and in the zinc hyperaccumulator *Arabidopsis halleri* (*FRO2*) (Becher *et al.*, 2004).

9.2 Local and Systemic Signaling

Reciprocal grafting studies with mutants harboring defects in iron uptake regulation and their respective wild-type plants strongly suggest that a long-range signal, communicating the shoot's iron status to the roots, participates in the control of iron stress responses (Grusak and Pezeshgi, 1996; Green and Rogers; 2004; for an extensive discussion see Curie and Briat, 2003, and Schmidt, 2003). This does not, *a priori*, rule out an autonomous sensing of iron by root cells. Several genes are induced after short (1 to 3 h) exposures to media lacking iron, potassium or phosphorous in both intact and decapitated plants, strongly supporting the existence of a local sensor (Wang *et al.*, 2002). Similarly, roots cultivated without a shoot are capable of inducing ferric reduction activity (Green and Rogers, 2004). Apparently, some of the responses to iron deficiency are mainly controlled by the local sensor. In intact tomato plants, transfer cells are induced upon iron deficiency which was temporarily and spatially associated with increased

proton extrusion. If iron was supplied to only a portion of the root system, high proton extrusion activity was recorded in roots supplied with iron, whereas transfer cells were mainly formed in Fe-deprived roots (Schmidt *et al.*, 2003). These results are in line with two iron sensing systems; one measuring the overall iron status in the shoot, whereas the other is sensing the iron level in a cell-autonomous way.

Signals received from the shoots have to be perceived by root tissues and transmitted to peripheral root cells, in which iron uptake takes place. As suggested by Bereczky *et al.* (2003), the vascular parenchyma may play an important role in the transduction of signals from the shoot. This is supported by expression of several genes potentially involved in iron signaling such as *LeNRAMP1*, *LeIRT2* and *FER* in this tissue.

9.3 Signal Components

FER is a root specific bHLH protein that appears to play a key role in iron homeostasis (Ling *et al.*, 2002). The T3238*fer* mutant is unable to install any of the responses to low Fe status and is heavily chlorotic at normal external iron levels (Brown *et al.*, 1971). *fer* roots do not develop extra root hairs and transfer cells in the rhizodermis upon Fe deficiency, pointing to an involvement of the *FER* gene in signal pathways leading to both morphological and physiological changes (Ling *et al.*, 2002; Schmidt *et al.*, 2000a). *FER* controls the expression of *LeIRT1*, *LeNRAMP1* and *LeFRO1* in roots but not in leaves, consistent with the idea of separate regulatory circuits. Similarly, the expression of *LeIRT2* and *LeNRAMP3* and the NA synthase gene *CHLN* is not dependent on the *FER* gene (Bereczky *et al.*, 2003; Li *et al.*, 2004). The direct downstream targets of FER have not yet been elucidated. A *fer* homolog has been recently isolated in *Arabidopsis* by three different groups Jakoby *et al.*, 2004; Colangelo and Guerinot, 2004; Yuan *et al*., 2004) and have been named *FIT1* (*Fe-Induced Transcription Factor 1*) and *FRU* (*FER*-like regulator of iron uptake), respectively. *fit1* mutant plants take up less iron than the wild-type and show differential expression of many iron-regulated genes. Interestingly, *FRO2* appears to be controlled by *FIT1* at the transcriptional level, whereas IRT1 is regulated at the level of protein accumulation (Colangelo and Guerinot, 2004).

Several genes with a potential involvement in iron deficiency signaling have been identified by microarray analysis in both Strategy I and Strategy II plants, but a specific role of these genes has not been elucidated (Thimm *et al.*, 2001, Wang *et al.*, 2002; Negishi *et al.*, 2002).

10. A RELIC OF EVOLUTION?

The concept of two different strategies in iron uptake of higher plants, originally defined by Römheld and Marschner in 1986, holds true also after nearly two decades and after molecular identification of a number of components of the iron uptake machinery has been achieved. In contrast to the original concept, the two strategies are apparently not mutually exclusive. Homologues of the *Arabidopsis* genes *FRO2* and *IRT1* that are regulated by iron availability have been found in rice, suggesting that a reductive iron uptake system may be active in Strategy II species in parallel to the PS-based iron acquisition Strategy (Bughio *et al.*, 2002; Gross *et al.*, 2003). Similarly, enhanced proton extrusion in response to iron deficiency is not restricted to Strategy I species; acidification of the nutrient solution was observed in Fe-deficient maize when nitrate was withheld from a part of the root system which masks net proton extrusion (Landsberg, 1979). In addition, at least in one Strategy II species epidermal cells revealed the characteristic build-up of transfer cells (Koyro, 1997). It thus appears that grasses have acquired the release of PS and subsequent uptake of the Fe-PS complex in addition to the reduction-based iron uptake system found in other species, possibly as an adaptation to soils high in pH. No major differences between the strategies appear to exist with respect to the intra- and intercellular transport of iron. Interestingly, a comparison between the genomes of *Arabidopsis* and rice revealed that in *Arabidopsis* the number of ZIP orthologs (15) is higher than that of YS orthologs (8), which is the opposite in rice, which has 18 YS members and 13 ZIP orthologs (Gross *et al.*, 2003). This may indicate that these gene families differ in their importance among the two strategies for iron acquisition. A key point in the development of Strategy II may lay in the evolution of NAAT, catalyzing the transamination of NA to form PS. Strategy I plants lack the ability to synthesize and secrete PS. To my knowledge, NAAT activity has not been observed in Strategy I species. How iron is taken up under conditions of sufficient iron availability has not been established. Low ferric reduction rates are evident in both species at sufficient iron levels, which opens the possibility that a reduction-based iron uptake serves for the iron acquisition under normal conditions.

With respect to the sensing and signaling of Fe deficiency, little information is available that allows for a comparison between Strategy I and Strategy II plants. No homologs of *FER* have been found by database research in rice, suggesting that at least some components of the transduction pathway are different (Ling *et al*., 2002). Interestingly, when the promoter sequence from the barley *Ids2* gene, encoding a dioxygenase involved in PS production, was fused to the *GUS* reporter gene, expression of the reporter

was promoted by iron-deficiency in the Strategy I plant tobacco (Yoshihara *et al.*, 2003). Sequences similar to that of two *cis*-acting elements of the *IDS2* promotor, IDE1 and IDE2, which are involved in Fe deficiency induced expression of *IDS2*, has been found in the promoters of *NAAT* genes of barley, *NAS* genes of rice, and in *AtFRO2, AtIRT1* and *AtNAS1* in *Arabidopsis*, suggesting that *cis*/*trans* systems are partially conserved in plants differing in their iron acquisition strategies (Suzuki *et al.*, 1999; Kobayashi *et al.*, 2003).

It thus appears that both the executing and signaling components of the Strategy I are partially conserved in grasses, and that a new, yet unknown sensing and signaling system has been evolved in the latter group. It has been estimated that 2 to 4% of the plants genome is involved in regulating nutrient homeostasis (Lahner *et al.*, 2003). Understanding the regulatory networks that lead to cellular i(r)on homeostasis will open strategies for developing plants with improved growth under iron-limited conditions and will help to understand the distribution of species in natural habitats.

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Chapter 12

PLANT HORMONES INFLUENCING IRON UPTAKE IN PLANTS

FRANCISCO J. ROMERA, CARLOS LUCENA and ESTEBAN ALCÁNTARA

Dpto. Agronomía, Escuela Técnica Superior de Ingenieros Agrónomos y de Montes, Universidad de Córdoba, Avda Menéndez Pidal s/n, Apdo. 3048, 14080 Córdoba, Spain. E-mail (F.J.R.): ag1roruf@uco.es

Abstract: Different experimental results based on split-root and grafting experiments suggest the involvement of systemic signals in the regulation of Fe deficiency stress responses by Strategy I plants. Until now, the nature of this (or these) systemic signal(s) is unknown, but several authors have proposed some plant hormones, or their precursors, as participants in this signalling process. Among the hormones that have received more attention are auxin and ethylene. Both of them can induce morphological changes similar to the ones induced by Fe deficiency, such as subapical root hairs and transfer cells, when applied to Fe-sufficient Strategy I plants. Furthermore, the addition of either auxin or ethylene inhibitors to Fe-deficient Strategy I plants can block some of these morphological changes. These results, and others obtained by using ethylene mutants and other experimental approaches, suggest that auxin and/or ethylene could be involved in the regulation of some of the morphological changes developed by Fe-deficient Strategy I plants. Since auxin can increase the production of ethylene, some of the effects of auxin could be mediated through ethylene. Auxin and ethylene have also been involved in the regulation of some of the physiological responses to Fe deficiency developed by Strategy I plants, such as acidification and ferric reductase activity. However, their participation in such regulation is more controversial. There are no studies relating hormones to Fe uptake in Strategy II plants, although ethylene and phytosiderophores share common precursors. Auxin and ethylene have also been involved in other nutrient deficiencies, namely those of P and K. In this chapter, we review existing evidence suggesting a role for auxin, ethylene and other hormones and signaling substances in the regulation of Fe deficiency stress responses and other aspects of Fe nutrition, and discuss the involvement of hormones in the responses to other nutrient deficiencies.

Key words: auxin; ethylene; ferric reductase; iron deficiency; root hairs

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1. INTRODUCTION

The influence of hormones on iron (Fe) uptake has been studied since the beginning of the 80's, with the pioneering works of Landsberg suggesting a role of auxin in the regulation of Fe deficiency stress responses in Strategy I plants (Landsberg 1981a, b, and c). Before that, there were a few studies relating hormone application to Fe chlorosis correction but often with contradictory results (reviewed in Landsberg, 1984). After the initial Landsberg's work, several authors have studied the role of hormones (mainly auxin and ethylene) in the regulation of some Fe deficiency stress responses in Strategy I plants (Landsberg, 1986 and 1996; Romera and Alcántara, 1994; Schmidt *et al.*, 2000b). To our knowledge there are no studies relating hormones to the regulation of Fe deficiency stress responses in Strategy II plants.

The roles of auxin and/or ethylene in the regulation of some of the Fe deficiency morphological responses in Strategy I plants, such as the development of subapical root hairs and transfer cells, are widely accepted (Landsberg, 1996; Romera and Alcántara, 2004; Schmidt and Bartels, 1996). However, the acceptance of the involvement of hormones in the regulation of Fe deficiency physiological responses, such as the enhanced ferric reductase activity and acidification is a controversial issue (Curie and Briat, 2003; Schikora and Schmidt, 2001; Schmidt *et al.*, 2003).

In this chapter, the existing evidence suggesting a role for hormones in the regulation of Fe deficiency stress responses and other aspects of Fe nutrition will be reviewed. This evidence is related mainly to Strategy I plants, although the possible relationship between both Strategies will be discussed.

2. EVIDENCE FOR SYSTEMIC SIGNALS INVOLVED IN THE REGULATION OF IRON DEFICIENCY STRESS RESPONSES BY STRATEGY I PLANTS

Bienfait *et al.* (1987) found that either sterile potato roots grown on a sucrose medium or potato roots occurring on tubers without sprouts were able to develop some of the Fe deficiency stress responses (i.e. enhanced ferric reductase activity, acidification, root hairs and transfer cells) when grown in a Fe-free medium. These results clearly show that the root itself can control the development of Fe deficiency stress responses (Bienfait *et al.*, 1987). Later on, working with sunflower plants consisting of an aerial part and two root systems (obtained by approach grafting), Romera *et al.*

(1992) showed that a root system growing without Fe was able to develop Fe deficiency stress responses even when the aerial part was green and with adequate Fe levels (the second root system was growing with Fe). This result also indicates that the root itself can control the development of Fe deficiency stress responses, even when the aerial part does contain enough Fe.

There are, however, several results suggesting that the control of Fe deficiency stress responses does not depend only on the root Fe content, as indicated by the two experiments described above, but that the regulation is far more complex and probably involves some signal(s) coming from the aerial part, or from other parts of the root. Landsberg (1981a, c; 1984) found that the Fe deficiency induced H^+ efflux can be prevented by removing the shoot apex of bean and sunflower plants, suggesting that a signal coming from the shoot apex (suggested to be auxin) would be involved in such a response. In the same way, Li *et al.* (2000) found that decapitation of the shoot apex in Fe-deficient bean plants decreased the ferric reductase activity, although it had no effect in Fe-deficient cucumber plants. In a split-root experiment similar to the one described in the previous paragraph, Romera *et al.* (1992) showed that a root system growing with Fe induced some of the responses (i.e. enhanced reductase activity and acidification) when the other root system was growing without Fe. To explain these results, Romera *et al.* (1992) proposed the existence of a systemic signal(s) that could move within the plant. Other split-root experiments have also shown that a root half growing with Fe can induce some responses, such as acidification (Schmidt *et al.*, 2003) and enhanced ferric reductase activity (Li *et al.*, 2000; Schikora and Schmidt, 2001), while the other root half is growing without Fe. Moreover, Vert *et al.* (2003) have recently shown that in *Arabidopsis* both the *FRO2* and the *IRT1* genes are induced in the +Fe-grown root half when the other root half is growing without Fe. This result clearly shows that systemic signal(s) affects the expression of both genes. Grusak (1995) showed that the ferric reductase activity of Fe-sufficient pea plants was modulated throughout the life cycle, which also suggests the existence of signal(s) derived from the shoot as modulators of the activity. By grafting the pea mutant *dgl* onto its wild type DGV, Grusak and Pezeshgi (1996) also showed that the reductase activity of the grafted plants was up-regulated, as occurred in non-grafted *dgl*, suggesting that the *dgl* shoot transmitted a signal that acted as a promoter of this response.

The above results suggest that under certain conditions, there are some signals that surpass the inhibitory effect of Fe on the expression of the responses. Furthermore, there are also results showing that Fe may be necessary for the formation or perception of such signal(s). In fact, under certain conditions, the application of Fe to Fe-deficient plants transitorily

stimulates some of the Fe deficiency stress responses. The supply of 40 μM FeEDTA to Fe-deficient bean plants always resulted in a sharp increase of the ferric reductase activity and an acidification burst (Sijmons and Bienfait, 1986). Similar results have been found in *Plantago* (Schmidt *et al.*, 1996), pea (Figure 12-6), and other plant species (Gogorcena *et al.*, 2004; Romera *et al.*, 1996a and references therein). It is important to note that in split-root experiments with *Arabidopsis* plants, the root half growing in the absence of Fe did not induce the expression of either *FRO2* mRNA or *IRT1* mRNA, which suggests that Fe is primarily required for the induction of both genes (Vert *et al.*, 2003).

From the above results, it can be concluded that roots themselves have the capacity to "decide" whether or not to induce Fe deficiency stress responses. However, to take such a "decision" roots need to integrate some signals coming from the aerial part, and perhaps from other parts of the roots.

3. ARE HORMONES THE SYSTEMIC SIGNALS?

To explain the results described in the previous paragraph, some authors have suggested the involvement of hormones as systemic signals in the regulation of Fe deficiency stress responses in Strategy I plants. To our knowledge, there are no hypotheses involving hormones in the regulation of Fe deficiency stress responses by Strategy II plants. Landsberg (1981a, b and c; 1984) and Römheld and Marschner (1981 and 1986) proposed that auxin is involved in the induction of some of the Fe deficiency stress responses, such as acidification, subapical root hairs and transfer cell formation. Landsberg (1981b) also showed that other hormones like gibberellins and cytokinins had no effect on root morphology, whereas ABA induced subapical root hairs but not transfer cells (Landsberg, 1996). ABA also inhibited root-driven medium acidification in Fe-deficient plants (Landsberg, 1981a and 1986). Romera and Alcántara (1994) and Romera *et al.* (1999) proposed that Fe deficiency causes an increase in ethylene production by roots, and that this hormone triggers Fe deficiency stress responses or, at least, some of them. Recently, Schmidt (2003) has suggested that NO (nitric oxide) is an attractive candidate for the translation of the Fe deficiency signal, but without presenting data to support this view. Other hormones, such as ABA, have been involved in other aspects of Fe nutrition, including the regulation of ferritin expression (Lobreaux *et al.*, 1993).

The two hormones that have received more attention are auxin and ethylene. At first, both could act as systemic signals, either *per se*, by their precursors, or by producing secondary messengers. As shown in the split-root

experiment of Figure 12-1, the application of either ACC (1-aminocyclopropane-1-carboxylic acid, an ethylene precursor, see Figure 12-2) or 2,4-D (2,4-dichlorophenoxyacetic acid, a synthetic auxin) to a root half induces subapical root hairs in that root half, but also in the other one. This implies that ACC, 2,4-D, or signals derived from them, can move within the plant. Since ethylene is a gas, it is not very mobile within the plant. In fact, the diffusion of ethylene in water is 10,000 times lower than in the air (Jackson, 1985). However, ACC can move in both the xylem (Finlayson *et al.*, 1991) and phloem (Morris and Larcombe, 1995). Auxin can move in the phloem both basipetally and acropetally (Friml and Palme, 2002).

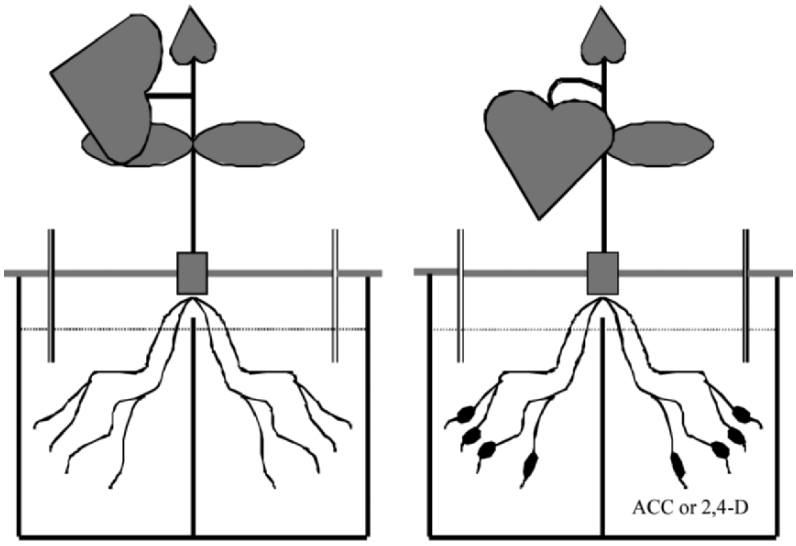

Figure 12-1. Scheme of a split-root experiment in cucumber plants showing that the addition of either ACC (10 μM) or 2,4-D (10 μM) to one root half induced subapical swelling with abundant root hairs in that root half but also in the other, untreated, root half. In all cases plants were grown with adequate Fe. Notice also the leaf epinasty induced by ACC or 2,4-D.

In the following paragraphs we will discuss the involvement of auxin, ethylene, and other hormones and signalling substances, in the regulation of the Fe deficiency stress responses in Strategy I and Strategy II plants, as well as in other aspects of Fe nutrition. We will also discuss the involvement of hormones in other nutrient deficiencies, to point out the similarities with Fe deficiency. Before that, we will briefly describe some characteristics of auxin, ethylene, and their interactions.

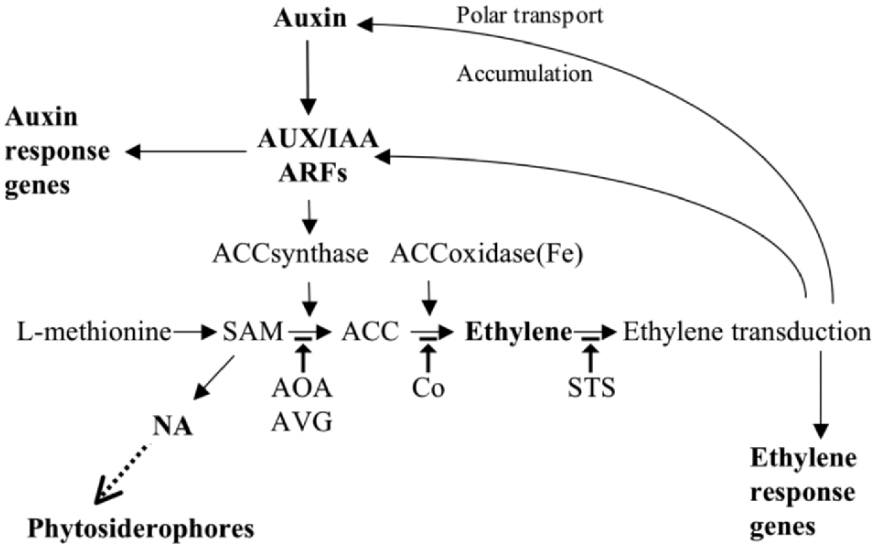

Figure 12-2. Schematic pathway of ethylene biosynthesis and its interactions with auxin. The steps at which AOA (aminooxyacetic acid), AVG (aminoethoxyvinylglycine), Co (cobalt) and STS (silver thiosulfate) act, as well as the pathway of NA and phytosiderophores biosynthesis. (⋯> : only in Strategy II plants; ⊤: inhibition) are also depicted.

4. AUXIN, ETHYLENE, AND THEIR INTERACTIONS

Auxin is a plant hormone involved in many plant growth metabolic processes, such as the regulation of growth, apical dominance, emergence of lateral roots, root hair differentiation, vascular differentiation, leaf epinasty, and others (Bhalerao *et al.*, 2002; Ljung *et al.*, 2001; Rahman *et al.*, 2002). The major endogenous auxin in plants is IAA (indole-3-acetic acid), although there are also other natural auxins, such as IBA (indole-3-butyric acid) (Bhalerao *et al.*, 2002). In addition, there are also synthetic auxins, such as 2,4-D and NAA (1-naphthaleneacetic acid). Auxin can be synthesized via a tryptophan-dependent or a tryptophan-independent pathway (Ljung *et al.*, 2002). Young developing leaves have the highest relative synthesis capacity, although the cotyledons are also capable of synthesizing IAA at a high rate. Expanding leaves and roots (mainly the root tip), have also the capacity to sinthesize IAA (Bhalerao *et al.*, 2002; Ljung *et al.*, 2001). In addition to the regulation of IAA biosynthesis, the level of this hormone can be controlled by conjugation and oxidation of the IAA molecule, and also by transport (Ljung *et al.*, 2001, 2002). During

conjugation, the side-chain of the active hormone is modified and forms conjugates with compounds such as sugars, amino acids, and polypeptides (Ljung *et al.*, 2002). The IAA molecule can also be oxidized via decarboxylative or non-decarboxylative reactions. The decarboxylative catabolism is mediated by a variety of peroxidases, often referred as 'IAA oxidases', although data obtained so far suggest that decarboxylative pathways are of minor importance in the regulation of endogenous auxin levels (Ljung *et al.*, 2002).

In relation to the transport of auxin, two main pathways have been described, including a fast, bidirectional (basipetal and acropetal directions), transport in the phloem and a slower, unidirectional, transport in various tissues (mainly cambium and adjacent cells), known as polar auxin transport (Friml and Palme, 2002). The main polar auxin transport stream runs from the shoot apex basipetally. In roots, the auxin stream flows acropetally towards the root tip, where part of the auxin is redirected backwards (basipetally) through the root epidermis to the elongation zone (Friml and Palme, 2002). In the last years, some genes probably involved in the redistribution of auxin within the root have been discovered, including the *AUX1* gene (belonging to the *AUX* gene family), possibly encoding an auxin influx carrier, and the *EIR1* gene (belonging to the *PIN* gene family), possibly encoding an auxin efflux carrier (Friml and Palme, 2002; Ljung *et al.*, 2002; Luschnig *et al.*, 1998). There are several inhibitors of the polar auxin transport, such as TIBA (2,3,5-triiodobenzoic acid), NPA (N-1-naphthylphthalamic acid), and CFM (2-chloro-9-hydroxyfluorene carboxylic acid-(9)-methylester), compounds which interfere with auxin efflux (Li *et al.*, 2000; Luschnig *et al.*, 1998). Recently, it has also been reported the existence of some compounds that act as inhibitors of auxin influx, such as CSI (chromosaponin I) and 1-NOA (1-naphthoxyacetic acid) (Rahman *et al.*, 2002).

In relation to its mode of action, it is known that auxin regulates gene expression through degradation of the AUX/IAA proteins (Figure 12-2). These short-lived nuclear proteins do not interact directly with the DNA but exert their regulatory activity through another group of proteins called auxin response factors (ARFs), which can act either as negative or positive regulators of gene transcription (Figure 12-2; Dharmasiri and Estelle, 2004; Gazzarrini and McCourt, 2003). There are mutants that overproduce auxin, such as *sur1* and *sur2* (Ljung *et al.*, 2002), and mutants altered in auxin signalling, such as *eir1* (auxin efflux carrier) and *aux1* (auxin influx carrier) (Dharmasiri and Estelle, 2004; Gazzarrini and McCourt, 2003; Luschnig *et al.*, 1998; Rahman *et al.*, 2002; Roman *et al.*, 1995).

Ethylene is a plant hormone involved in many plant processes, including seed germination, root hair development, root nodulation, flower senescence,

abscission, and fruit ripening (Lynch and Brown, 1997; Wang *et al.*, 2002). The production of ethylene is tightly regulated by internal signals, and in response to biotic (e.g. pathogen attack) and abiotic stresses, such as wounding, mechanical stress, hypoxia, excess of ozone, chilling, freezing, and nutritional disorders (He *et al.*, 1992; Lynch and Brown, 1997; Morgan and Drew, 1997; Wang *et al.*, 2002). Stress usually promotes ethylene production (Morgan and Drew, 1997; Yang and Hoffman, 1984).

Ethylene is synthesized from L-methionine via the pathway shown in Figure 12-2, which also includes some of the inhibitors of ethylene biosynthesis and action (Yang and Hoffman, 1984; Wang *et al.*, 2002). ACC can also be conjugated to form MACC (N-malonyl-ACC) (Yang and Hoffman, 1984; Finlayson *et al.*, 1996). Ethylene effects can be regulated through its biosynthethic pathway (synthesis, conjugation and conversion of ACC; Finlayson *et al.*, 1996), and also via differential expression of its receptors (Klee, 2004). Ethylene is produced in all parts of higher plants (Yang and Hoffman, 1984). In roots, it has been shown that root tips have higher ethylene production rates than middle and basal root parts (Finlayson *et al.*, 1996).

The mode of action of ethylene is still not fully understood, but in recent years there have been considerable advances, mainly based on the study of *Arabidopsis* ethylene mutants. Several ethylene insensitive mutants have been identified, such as *etr1*, *ers1*, *ein4*, *ein2* and *ein3*. The *ETR1*, *ERS1* and *EIN4* genes encode ethylene receptors and the *EIN2* and *EIN3* genes encode proteins involved in the ethylene signalling pathway (Guo and Ecker, 2004; Wang *et al.*, 2002). On the other hand, *ctr1* is a mutant that constitutively activates most of the responses to ethylene (i.e. triple response), as if the hormone was always present (Guo and Ecker, 2004; Wang *et al.*, 2002). There are also mutants that overproduce ethylene, such as *eto1* (Roman *et al.*, 1995). Based on epistatic analyses of different *Arabidopsis* ethylene mutants, a transduction pathway for ethylene has been proposed, in which CTR1 acts downstream of ETR1, ERS1 and EIN4 (and other receptors: ETR2 and ERS2), and EIN2 and EIN3 acts downstream of CTR1 (Gazzarrini and McCourt, 2003; Guo and Ecker, 2004; Wang *et al.*, 2002):

Ethylene → ETR1, EIN4, …⊣‖CTR1 ⊣‖ → → → EIN2 → EIN3 → responses

In this pathway, ethylene receptors are negative regulators of the ethylene response pathway, i.e. they repress ethylene responses in the absence of ethylene. Similarly, CTR1, which has homology to the Raf family of protein kinases, is a negative regulator of downstream signalling events (Guo and Ecker, 2004; Wang *et al.*, 2002). Recent data suggest that the ethylene signal

transduction pathway is more complex than the lineal transduction pathway depicted above, and that there are branches and cross-talks with other hormones such as jasmonic acid (Guo and Ecker, 2004).

It is difficult to assign the regulation of a process to an unique hormone. Some hormones affect the biosynthesis of others, and also hormones act through complex signalling pathways, in which there are cross-talks among them (Gazzarrini and McCourt, 2003; Hansen and Grossmann, 2000; Wang *et al.*, 2002). Auxin and ethylene can interact at different levels (Figure 12-2). Auxin can enhance ethylene production by affecting the ACC synthase activity (Hansen and Grossmann, 2000; Swarup *et al.*, 2002; Yang and Hoffman, 1984). On the other hand, ethylene could affect auxin accumulation and polar auxin transport (Swarup *et al.*, 2002). Recently, it has been found that ethylene can regulate the transcription of *AUX/IAA* and *ARF* genes (Jones *et al.*, 2002; Pech *et al.*, 2004). The complex interactions between auxin and ethylene are illustrated by the fact that there are several *Arabidopsis* mutants affected in the signalling of both hormones, such as *aux1*, *axr1*, *axr2*, and *eir1* (Roman *et al.*, 1995; Swarup *et al.*, 2002).

5. ROLE OF AUXIN AND ETHYLENE ON IRON DEFICIENCY STRESS RESPONSES BY STRATEGY I PLANTS

Landsberg (1981a, b, c; 1984; 1996) and Römheld and Marschner (1981, 1986) proposed that Fe deficiency increases the levels of auxin, either coming from the Fe-deficient shoot apex or accumulated in the root due to a low "IAA oxidase" activity, and that this hormone triggers some of the Fe deficiency stress responses in Strategy I plants, such as the subapical root hairs and transfer cells. As described by Ljung *et al.* (2002), "IAA oxidases" have little effect in mantaining IAA levels, which suggests that the increase of auxin levels in roots should be due to other mechanisms, such as an increase in IAA coming from Fe-deficient shoot apex (Landsberg, 1981a). It has been reported that Fe-deficient sunflower roots produced higher levels of auxin than the Fe-sufficient ones (Römheld and Marschner, 1986).

As an alternative to the auxin hypothesis, Romera and Alcántara (1994) proposed that Fe deficiency causes an increase in ethylene production by roots, and that this hormone triggers Fe deficiency stress responses or, at least, some of them. More recently, it has been shown that Fe-deficient roots of several Strategy I plants produced more ethylene than Fe-sufficient ones (Figure 12-3; Romera *et al.*, 1999; Waters and Blevins, 2000). The highest ethylene production occurred 3-9 days after removing Fe from the nutrient solution (depending on the species), and was parallel to the induction of

several Fe deficiency stress responses, such as enhanced ferric reductase activity, the formation of subapical root hairs and acidification of the medium (Figure 12-3; Romera *et al.*, 1999; Waters and Blevins, 2000).

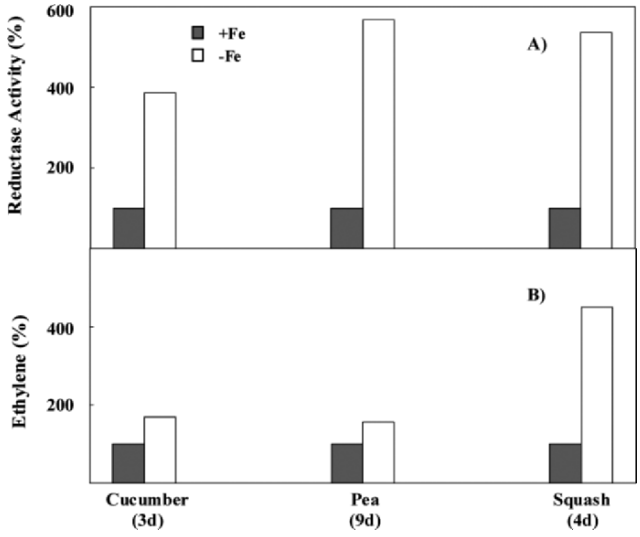

Figure 12-3. Ferric reductase activity (A) and ethylene production by roots (B) of Fe-sufficient (+Fe) and Fe-deficient (-Fe) cucumber, pea, and squash plants. Data are expressed as percentage of values in Fe-deficient plants as compared with the Fe-sufficient ones. Within parentheses are the number of days in the -Fe treatment. (Reelaborated from Romera *et al.*, 1999; and Waters and Blevins, 2000).

Here, we will describe several experimental results that suggest a role of auxin and ethylene in the regulation of some of the morphological responses (formation of subapical root hairs and transfer cells) and some of the physiological responses (e.g. enhanced ferric reductase activity, acidification of the medium or flavin excretion) to Fe deficiency developed by Strategy I plants. The experimental results showing an involvement of ethylene in the regulation of Fe deficiency stress responses have been reviewed recently (Romera and Alcántara, 2004).

5.1 Morphological responses

The involvement of auxin in the regulation of morphological responses to Fe deficiency is based on several experimental results. First, the addition of auxin (IAA or 2,4-D) to Fe-sufficient plants induced the development of subapical swelling with abundant root hairs and transfer cells similar to the ones developed under Fe deficiency (Figure 12-4a; Landsberg, 1981b, 1984,

1996; Schmidt and Bartels, 1996; Schmidt *et al.*, 2000a, 2003). Second, the addition of TIBA (inhibitor of polar auxin transport) to Fe-deficient plants inhibited the development of subapical root hairs (De la Guardia *et al.*, 1988).

Similarly to auxin, the addition of ACC or ethephon (an ethylene releasing substance) to Fe-sufficient plants also promoted the development of both subapical root hairs and transfer cells in roots of several Fe-sufficient Strategy I plants (Figure 12-4c; Romera and Alcántara, 1994, 2004; Romera *et al.*, 1997, 1999; Schikora and Schmidt, 2002a and b; Schmidt *et al.*, 1999; Schmidt *et al.*, 2000a, 2003). Experiments performed with some ethylene mutants have also shown that ethylene is involved in the regulation of subapical root hairs. The *Arabidopsis* mutants *etr1* and *ein2* did not develop subapical root hairs either under Fe-deficiency or upon ACC treatment, whereas the wild-type *Columbia* did (Romera and Alcántara, 2003; Romera *et al.*, 1997). Similarly, the soybean mutant *etr1* and the *Medicago truncatula* mutant *sickle*, both insensitive to ethylene, did not develop subapical root hairs upon ACC treatment, while their respective wild-types did (Penmetsa and Cook, 1997; Romera and Alcántara, 2004; Schmidt *et al.*, 1999). In contrast, the *Arabidopsis* mutant *ctr1* developed subapical root hairs even under Fe-sufficient conditions (Romera and Alcántara, 2004). These results suggest that, in *Arabidopsis*, the genes *ETR1*, *EIN2*, and *CTR1* are involved in the development of subapical root hairs by Fe-deficient plants.

The alteration of the tomato mutant *fer* is also probably related to ethylene. This mutant does not develop either transfer cells or subapical root hairs when grown under Fe deficiency (Bienfait, 1987, 1988). However, it developed both morphological responses when treated with ACC (Figure 12-4c; Romera *et al.*, 1997; Schmidt *et al.*, 2000a). Since the *FER* gene probably codes for a bHLH transcription factor (Ling *et al.*, 2002), the possibility exists that this transcription factor could interact somehow with ethylene.

In contrast to the effects of ACC and ethephon in Fe-sufficient plants, the addition of ethylene inhibitors, such as Co, STS, AOA or AVG (see Figure 12-2), to Fe-deficient plants inhibited the development of subapical root hairs and transfer cells (Figure 12-4b; Landsberg, 1982; Romera and Alcántara, 1994, 2004).

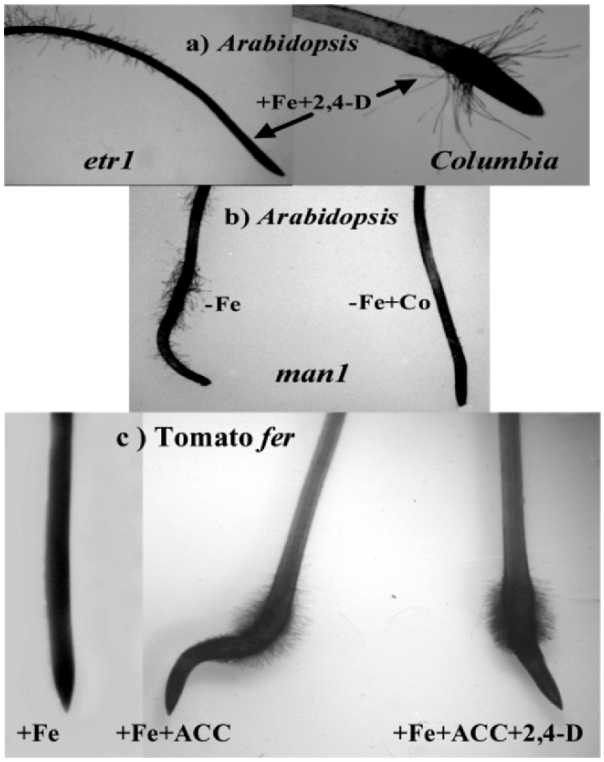

Figure 12-4. Effect of 2,4-D, ACC and Co on the development of subapical root hairs by wild-type and mutant roots of *Arabidopsis*, and the tomato mutant *fer*. Plants were grown in Fe-sufficient (a and c) or Fe-deficient conditions (b). ACC (at 0.5 µM), 2,4-D [at 0.5 µM (*fer*), 1 µM (Columbia), and 50 µM (*etr1*)], or $CoSO_4$ (25 µM) were added for 24 h before excising roots and staining them with toluidine blue (0.05% w/v).

From the above results, it is clear that both auxin and ethylene could induce morphological responses similar to those induced by Fe deficiency. Nonetheless, since auxin can enhance ethylene production (Figure 12-2; Hansen and Grossmann, 2000; Swarup *et al.*, 2002; Yang and Hoffman, 1984), it is also possible that the effect of auxin on morphological responses could be mediated through ethylene. This possibility is supported by some experimental results. First, the addition of 2,4-D to Fe-sufficient cucumber plants induced the development of subapical root hairs, but this did not occur when 2,4-D was applied along with an ethylene inhibitor, like Co or STS (Romera *et al.*, 1997). Second, the addition of 2,4-D induced subapical root hairs in the *Arabidopsis* wild-type cultivar *Columbia* but not in the ethylene insensitive mutants *etr1* (Figure 12-4a) and *ein2* (Romera and Alcántara, 2004). These results suggest that auxin could induce subapical root hairs *via*

ethylene. However, recent results from Takahashi *et al.* (2003) suggest that ethylene promotes root hair initiation by increasing the sensitivity of the hair-forming cells to auxin, implying that both hormones are required for the formation of root hairs. So, it is probable that under Fe deficiency both hormones could participate in the induction of the morphological changes. ACC induced subapical root hairs in the tomato mutant *fer* but with an exaggerated curvature of the subapical region, that dissapeared when ACC was applied along with 2,4-D (Figure 12-4c). This result clearly suggests that there is an interplay between both auxin and ethylene in the development of the subapical region of the root.

5.2 Physiological responses

The involvement of auxin in the regulation of physiological responses to Fe deficiency is mainly based on several experimental results obtained by using auxin transport inhibitors. The application of TIBA or CFM (inhibitors of polar auxin transport) to Fe-deficient plants markedly postponed the onset of medium acidification and inhibited ferric reductase activity (De la Guardia *et al.*, 1988; Landsberg, 1981a and c, 1984; Li *et al.*, 2000). In the same way, decapitation of the shoot apex to Fe-deficient plants also prevented the onset of some physiological responses, such as acidification and enhanced ferric reductase activity, which has been attributed to the elimination of the auxin flux coming from the shoot apex (Landsberg, 1981a and c, 1984; Li *et al.*, 2000).

The results obtained after application of auxin to Fe-sufficient plants, in an attempt to induce some of the physiological responses, have been less conclusive. Schmidt (1994) found that 2,4-D addition to either Fe-sufficient or Fe-deficient *Plantago* plants did not significantly induce root ferric reductase activity. Similarly, the addition of 2,4-D to Fe-sufficient tomato plants did not induce acidification (Schmidt *et al.*, 2003).

Evidence for the involvement of ethylene in the regulation of some of the physiological responses to Fe deficiency also derives from results obtained with ethylene inhibitors and precursors. The addition of inhibitors of ethylene synthesis (Co, AOA, AVG) or action (STS) to several Fe-deficient plants, at different concentrations depending on the species, inhibited the induction of most of their physiological responses to Fe deficiency, such as root ferric reductase activity (Figures. 12-5 and 12-6), acidification, and flavin excretion (Romera and Alcántara, 2004). The inhibitory effect of AVG and Co on ferric reductase activity was reversed by the addition of ACC (Figure 12-6; Romera and Alcántara, 1994) and Fe (Figure 12-6; Romera *et al.*, 1996a), respectively, which clearly suggests that the inhibitory effect was not due to general toxic effects. It is worth mentioning

that in both the pea mutant *brz* and the tomato mutant *chloronerva* the ferric reductase activity, which is constitutively up-regulated, was also drastically inhibited by the addition of ethylene inhibitors (Figure 12-5; Romera *et al.*, 1996b). It should be noted that the *brz* mutant exhibits a low root nodulation ability, which is partly restored upon treatment with the ethylene inhibitors AVG or Ag^{+} (Guinel and LaRue, 1992). Since ethylene inhibits root nodulation (Lynch and Brown, 1997), it is tempting to suggest that this mutant has some alterations in ethylene metabolism or perception (Guinel and LaRue, 1992; Romera *et al.*, 1996b). Recently, our group has found, in cooperation with Dr. Waters (University of Massachusetts, USA), that ethylene inhibitors block the transcription of both the *FRO* (ferric reductase) and the *IRT* (iron transporter) genes (Lucena *et al.*, manuscript in preparation). These results clearly suggest a role for ethylene in the regulation of both physiological responses.

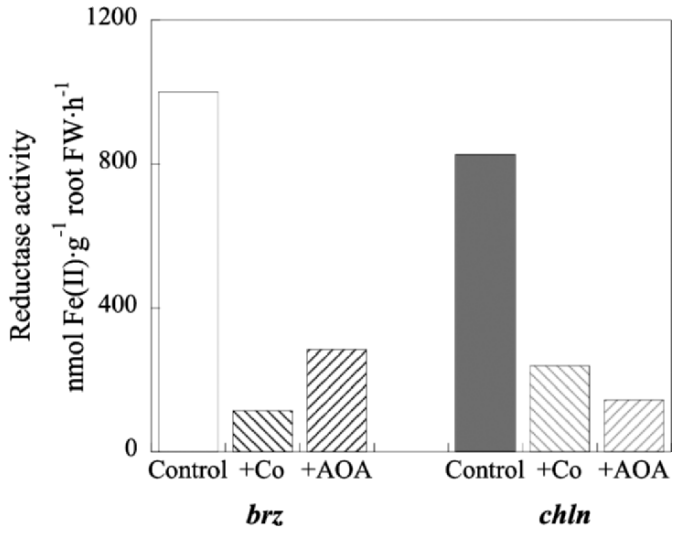

Figure 12-5. Effect of Co and AOA on the ferric reductase activity of Fe-deficient plants of the pea mutant bronze (*brz*) and in Fe-sufficient plants of the tomato mutant chloronerva (*chln*). Pea plants were grown in nutrient solution without Fe while tomato plants were grown in nutrient solution with 10 µM FeEDDHA. $CoSO_4$ [at 3 µM (*brz*) or 5 µM (*chln*)] or AOA [at 20 µM (*brz*) or 5 µM (*chln*)] were applied to some of the plants during the last 2 days before determining ferric reductase activity. (Reelaborated from Romera *et al.*, 1996b).

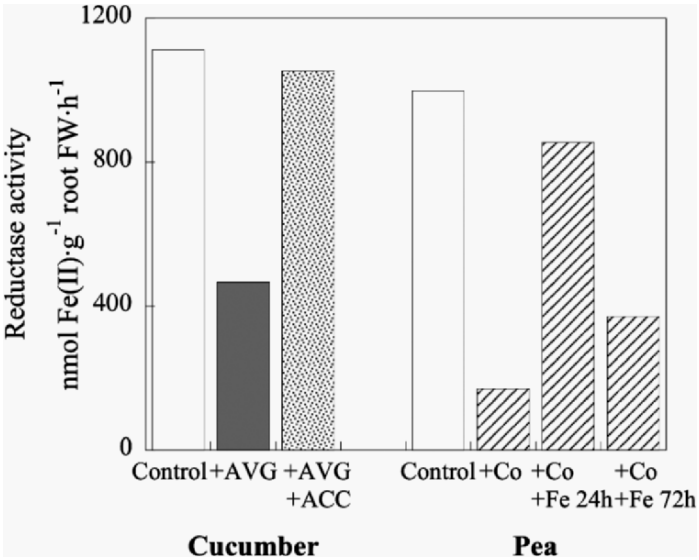

Figure 12-6. Effect of AVG-ACC and of Co-Fe on the ferric reductase activity of Fe-deficient cucumber and pea plants. Cucumber plants were grown in nutrient solution without Fe for the last 4 days, and AVG (at 10 µM) or AVG (at 10 µM)+ACC (at 1 µM) were applied to some of the plants during the last 2 days before determining ferric reductase activity. Pea plants were grown in nutrient solution without Fe, and $CoSO_4$ (at 3 µM) was applied to some of the plants on day 12. On day 13, Fe (at 20 µM) was added to some of the Co-treated plants. Ferric reductase activity was determinded on day 14 (Control, +Co, and +Co+Fe 24h) and on day 16 (+Co+Fe 72h). (Reelaborated from Romera and Alcántara, 1994; and Romera *et al.*, 1996a).

In contrast to ethylene inhibitors, which blocked the induction of Fe deficiency stress responses in Fe-deficient plants, the addition of ethylene precursors to Fe-sufficient plants induced several physiological responses to Fe deficiency, such as enhanced ferric reductase activity. The addition of ACC to Fe-sufficient cucumber, pea, sugar beet, *Arabidopsis*, and tomato plants enhanced their ferric reductase activity by about 2- to 5-fold (Romera and Alcántara, 1994, 2003, 2004; Romera *et al.*, 1999, 2003). Similarly, the addition of ethephon to Fe-sufficient *Plantago* plants also enhanced their ferric reductase activity about 2-fold (Romera *et al.*, 1999). In both cases, the enhanced reductase activity was located in the subapical regions of the roots, where the formation of root hairs was induced, as occurred in Fe-deficient plants (Romera and Alcántara, 1994 and 2004). When ACC was applied along with Fe deficiency to cucumber, pea, carrot, and sugar beet plants, for 24 h, it also enhanced ferric reductase activity by about 2-fold (Romera and Alcántara, 2004; Romera *et al.*, 2003). In the latter case, the results were easier to reproduce than with Fe-sufficient plants, in which, sometimes, the addition of ACC enhanced the reductase activity by more than 5- to 10-fold,

while other times it had no effect. The latter results suggest that the induction of the ferric reductase activity does not depend on ethylene alone, as it will be discussed later.

Treating Fe-sufficient plants with ACC induce significant acidification of the nutrient solution only in exceptional cases. However, evidence of acidification in the subapical regions of the root was obtained in agar plates with bromocresol purple (data not shown).

The use of mutants to demonstrate a role for hormones in the regulation of physiological responses, such as ferric reductase activity, has been problematic and confusing. So far, all mutants tested (including auxin insensitive, ethylene insensitive and constitutive, ABA insensitive, and cytokinin insensitive) enhanced their ferric reductase activity under Fe deficiency (Romera and Alcántara, 2003 and 2004; Schmidt *et al.*, 2000b). This has led some authors to conclude that hormones are not involved in the regulation of physiological responses to Fe deficiency, and that the morphological and physiological responses are independently regulated (Schikora and Schmidt, 2001; Schmidt *et al.*, 2000b). For us, such a conclusion could be inadequate because the new knowledge about hormone signalling and ferric reductase regulation shows that both processes are more complex than previously thought. First, the ferric reductase, as well as the iron transporter, are regulated at both transcriptional and post-trancriptional levels (Connolly *et al.*, 2002 and 2003). This means that, even when a gene is expressed, its product could be not detected due to the post-transcriptional regulation. Second, the complex signalling networks among the different hormones (Gazzarrini and McCourt, 2003; Hansen and Grossmann, 2000; Wang *et al.*, 2002) imply that most of the processes regulated by hormones do not depend on linear, unbranched transduction pathways. Perhaps, this is the reason of the difficulty to find a hormone mutant impaired in its capacity to induce some of the physiological responses to Fe deficiency.

A conclusion derived from the experiments with ethylene mutants is that subapical root hairs and ferric reductase activity are not regulated by ethylene the same way, as first proposed by Romera *et al.* (1997). In *Arabidopsis*, subapical root hairs are not developed in the ethylene insensitive mutants *etr1* and *ein2* either under Fe deficiency or upon ACC treatment, and are constitutively presented in the ctr1 mutant, even under Fe- sufficient conditions. On the other hand, ferric reductase activity is induced in all *Arabidopsis* mutants under Fe deficiency (Romera and Alcántara, 2003, 2004; Schmidt *et al.*, 2000b). Additionally, in the mutant *etr1* ferric reductase activity is induced upon ACC treatment and inhibited upon ethylene inhibitors treatment (Romera and Alcántara, 2003, 2004). Therefore, in *Arabidopsis* ethylene could participate in the regulation of subapical root hairs through a transduction pathway including the *ETR1*,

EIN2, and *CTR1* genes, which agrees with the models proposed by Masucci and Schiefelbein (1996) and Schmidt and Schikora (2001). For the regulation of ferric reductase activity and other physiological responses, ethylene could act through other transduction pathways (Romera and Alcántara, 2000, 2003; Romera *et al.*, 1997).

The evidence obtained by using ethylene inhibitors and precursors and ethylene mutants suggests that ethylene is involved in the regulation of most of the Fe deficiency stress responses, such as subapical root hairs, transfer cells, ferric reductase activity, iron transporter, acidification, and flavin excretion (Romera and Alcántara, 2004). There are also other responses to Fe deficiency, such as the release of phenolics (Römheld and Marschner, 1983), whose induction has not been studied in relation to ethylene. However, some indirect studies suggest that the biosynthesis of some phenolics, like caffeic, coumaric and ferulic acids, could be stimulated by ethylene through an increase in the activity of the phenylalanine ammonia lyase (Rhodes and Wooltorton, 1973).

It is possible that the different responses to Fe deficiency could be regulated by ethylene in different ways. First, the different responses could be induced upon different ethylene concentrations or sensitivities. Second, ethylene could regulate different responses through different transduction pathways. Third, ethylene could regulate different responses by acting in coordination with other signals, such as auxin, jasmonic acid and others. Results obtained so far suggest that the morphological responses are easier to induce upon hormone treatment (auxin or ethylene) than the physiological responses. This probably means that the regulation of the physiological responses is much more complex. Fourth, the regulation of the responses in different plant species could have specific and different characteristics.

In relation to auxin, the evidence found so far suggests its probable participation in the formation of subapical root hairs and transfer cells in conjunction with ethylene. In other responses, it is possible that auxin could participate by affecting ethylene levels. In some experiments we have found an increase of ferric reductase activity upon 2,4-D treatment to Fe-sufficient plants, but we have never found such a increase when we applied 2,4-D along with ethylene inhibitors such as Co or STS (unpublished results).

6. ROLE OF HORMONES ON IRON DEFICIENCY STRESS RESPONSES BY STRATEGY II PLANTS

Strategy II plants enhance the production of phytosiderophores under Fe deficient conditions (Ma and Nomoto, 1996; Mori, 1999) but, to our knowledge, no signals other than Fe have been involved in their regulation.

Nonetheless, some similarities could be found between Strategy I and Strategy II plants. As shown in Figure 12-2, both ethylene and phytosiderophores are synthesized from L-methionine. So, it is likely that Strategy I and Strategy II plants may share some common elements in their responses to Fe deficiency. Some experimental results do not support a role of ethylene in the regulation of Fe deficiency stress responses by Strategy II plants. First, roots from several Fe-deficient Strategy II plants did not produce more ethylene than Fe-sufficient ones, as it occurrs in Strategy I plants (Romera *et al.*, 1999), although in some experiments it has been shown that Fe-deficient sorghum plants (Strategy II) produced much more ethylene than Fe-sufficient ones (Morgan and Hall, 1962). Nonetheless, in that study ethylene determination was done when plants were very chlorotic and, consequently, the higher ethylene production could be due to indirect effects. Second, the addition of ACC to barley seedlings did not increase their production of phytosiderophores (Welch *et al.*, 1997).

There are, however, some experimental results suggesting that both Strategy I and Strategy II plants could share some common signals in the induction of their Fe deficiency stress responses. Higuchi *et al.* (2001) found that the barley *HvNAS1* nicotianamine synthase [involved in NA (nicotianamine) synthesis; see Figure 12-2] promoter-*gus* fusion gene was induced in transgenic tobacco plants under Fe deficiency. Similarly, Yoshihara *et al.* (2003) showed that the Fe deficiency responsive gene, *Ids2*, of barley was induced under Fe deficiency in transgenic tobacco plants. These results clearly indicate that some signals involved in the induction of Fe deficiency stress responses by Strategy I plants could be recognized by the promoters of the genes involved in Fe deficiency stress responses by Strategy II plants. Curiously, the promoter region of some *NAS* genes of *Arabidopsis thaliana* has an ethylene-responsive element (Suzuki *et al.*, 2001).

7. HORMONES AND OTHER ASPECTS OF IRON NUTRITION

Besides Fe uptake, some hormones and signalling substances have been involved in other aspects of Fe nutrition. ABA and NO have been related to the transcription of some ferritin genes induced by iron excess (Lobreaux *et al.*, 1993; Murgia *et al.*, 2002). Graziano *et al.* (2002) have also shown that NO affects the availability and/or delivery of metabolically active Fe within the plant. The authors found that NO treatment did not increase Fe content in plant organs, suggesting that root Fe uptake is not enhanced (Graziano *et al.*, 2002). According to the above results, NO could play a role

in Fe remobilization within the plant and in Fe homeostasis. In some processes like fruit ripening, an inverse relationship between NO and ethylene production has been found (Leshem and Pinchasov, 2000). This result suggests that both gaseous substances interact somehow in this process. It is possible that they could interact similarly in relation to Fe nutrition.

8. ROLE OF HORMONES ON OTHER NUTRIENT DEFICIENCIES

Although hormones have been related to mineral deficiencies in many studies (e.g. Battal *et al.*, 2003; Kuiper *et al.*, 1989), only in some of them hormones have been involved in the responses developed to alleviate those nutrient deficiencies. However, it is clear that many nutritional responses must involve the transmission of long-distance signals between the shoot and the root (Forde, 2002). The role of long-distance signals coming from the shoot in regulating gene expression in the root has been demonstrated for the response to several nutrient deficiencies, such as phosphorus (Burleigh and Harrison, 1999), sulfur (Lappartient *et al.*, 1999) and iron (Grusak and Pezeshgi, 1996). Here, we will briefly describe the responses to some nutrient deficiencies where hormones have been involved.

Plant responses to phosphorus (P) deficiency have many similarities with the response to Fe deficiency. Under both nutrient deficiencies, morphological and physiological changes are induced in the roots as a strategy to increase the mobilization and absorption of these elements. The regulation of the responses to P deficiency is complex and not well known, but results from different authors suggest the involvement of several hormones such as auxin, ethylene and cytokinins (Abel *et al.*, 2002; Franco-Zorrilla *et al.*, 2004; Neumann *et al.*, 2000).

Increase in root-hair length and density (López-Bucio *et al.*, 2002; Zhang *et al.*, 2003), induction of transfer cells (Schikora and Schmidt, 2002a) and excretion of organic acids, phenolics and protons (Dinkelaker *et al.*, 1995; Neumann and Römheld, 1999; Yan *et al.*, 2002; Watt and Evans, 1999) are found in both P and Fe deficiencies. The formation of proteoid and cluster roots is a characteristic response to P deficiency in some plant species (Dinkelaker *et al.*, 1995; Neumann *et al.*, 2000; Watt and Evans, 1999), which can also be induced by Fe deficiency (McCluskey *et al.*, 2004; Watt and Evans, 1999; Zaïd *et al.*, 2003). The different responses are not synchronized and it is generally accepted that they could be regulated by different pathways.

Auxin and ethylene have been implicated in the morphological responses to P deficiency, and cytokinins in both morphological and

physiological responses (Abel *et al.*, 2002; Franco-Zorrilla *et al.*, 2004; Neumann *et al.*, 2000). He *et al.* (1992) showed that P-deficient corn plants had enhanced sensitivity to ethylene in their roots. Increases in the concentration of ethylene and auxin have been reported in P deficient roots (Borch *et al.*, 1999; Gilbert *et al.*, 2000). Exogenous applications of auxins, or ACC, to P sufficient plants produced morphological changes in the roots similar to those found in P deficient plants (Gilbert *et al.*, 2000; López-Bucio *et al.*, 2002; Neumann *et al.*, 2000; Schmidt and Schikora, 2001; Zhang *et al.*, 2003). In addition, the morphological changes can be inhibited in P deficient plants by the application of either polar auxin transport inhibitors or ethylene inhibitors (Gilbert *et al.*, 2000; López-Bucio *et al.*, 2002; Zhang *et al.*, 2003), although this inhibitory effect was not always found (Gilbert *et al.*, 2000; Schmidt and Schikora, 2001). In *Arabidopsis*, auxin and ethylene antagonists reduced the extra root-hair density promoted by Fe deficiency, but did not counteract this response in P deficient plants, which suggest differences in the signal pathways involved in both stresses (Schmidt and Schikora, 2001).

Results obtained with auxin and ethylene related mutants show that many of them conserve the capacity to develop morphological changes in their roots under P deficiency (López-Bucio *et al.*, 2002; Schmidt and Schikora, 2001; Zhang *et al.*, 2003). These results have led to the conclusion that these hormones are not involved in the regulation of the morphological responses. However, the results described above, in experiments with application of the hormones or their antagonists, clearly support their involvement. Possibly, this apparent contradiction could be clarified with a more detailed knowledge of the signal pathways.

Hormones have also been involved as long-distance signals in relation to nitrogen (N) nutrition (Forde, 2002; Gawronska *et al.*, 2003; Takei *et al.*, 2001). Studies with tobacco have revealed a strong negative correlation between the shoot NO_3^- content and the allocation of resources to root growth and branching (Stitt and Feil, 1999). Forde (2002) suggested that NO_3^- accumulation in the shoot might negatively regulate root branching by inhibiting auxin biosynthesis or its transport to the root. On the other hand, Takei *et al.* (2001) observed that NO_3^- resupply to N-deprived roots rapidly stimulated cytokinin biosynthesis and transport to the shoot. This observation suggests that cytokinins are long-distance signals mediating the molecular response to changes in NO_3^- availability (Takei *et al.*, 2001). Given the known role of cytokinins as regulators of growth and cell division (D'Agostino and Kieber, 1999), the NO_3^--dependent changes in cytokinins production in roots could provide a mechanism for regulating leaf expansion in response to short-term fluctuations in NO_3^- availability.

The role of hormones on N nutrition is also related to the induction of nitrate reductase. This enzyme is able to reduce NO_3^- to NO_2^- and also Fe^{3+} to Fe^{2+} (Campbell and Redinbaugh, 1984). Schmerder and Borriss (1986) found, in *Agrostemma githago* embryos, that nitrate reductase activity was synergistically enhanced by cytokinin and ethylene application. Similarly, Vuylsteker *et al.* (1997) found enhanced nitrate reductase activity in chicory roots upon cytokinin and auxin treatments. On the other hand, He *et al.* (1992) showed that N-deficient corn plants had enhanced sensitivity to ethylene in their roots.

In relation to other nutrient deficiencies, Barker and Corey (1988) showed that tomato plants deficient in K, Ca, or Mg produced more ethylene than healthy ones. However, in this experiment ethylene was measured when plants showed severe symptoms of deficiency (Barker and Corey, 1988), and the higher ethylene production could be due to indirect effects more than specific effects of the deficiencies. Nonetheless, ethylene and jasmonic acid have been recently shown to be involved in the response to K^+ deficiency (Armengaud *et al.*, 2004; Shin and Schachtman, 2004). Shin and Schachtman (2004) found enhanced ethylene production in *Arabidopsis* plants upon K^+ deprivation, accompanied with changes in root morphology. In addition, they found enhanced expression of some genes involved in ethylene production and transduction, including ACC oxidase genes and ethylene-responsive transcription factors, and in K^+ uptake transporters (Shin and Schachtman, 2004). Besides ethylene, Shin and Schachtman (2004) found an increase in reactive oxygen species (ROS) upon K^+ deprivation, and suggested that both ethylene and ROS could be involved in the responses to K^+ deficiency. It is probable that ROS and ethylene could also be involved in the responses to other nutrient deficiencies, such as Fe and P, since ROS also accumulated under Fe deficiency (Ranieri *et al.*, 2003) and P deficiency (Juszczuk *et al.*, 2001).

9. CONCLUDING REMARKS

The existing results support the involvement of auxin and ethylene in the regulation of some of the Fe deficiency stress responses by Strategy I plants. The regulation of subapical root hairs in Fe-deficient plants could be mediated by ethylene and auxin according to the following transduction pathway:

Fe-deficiency → ethylene → ETR1 ⊣ CTR1 ⊣ → → EIN2 → → auxin → subapical root hairs.

For the regulation of the ferric reductase activity, ethylene could act through a different, still unknown transduction pathway:

Fe-deficiency → ethylene and other signals → ? → ? → FRO mRNA → posttranscriptional regulation → ferric reductase.

The regulation of other Fe deficiency stress responses, such as transfer cells, flavin excretion, acidification, and iron transporters, have been less studied, but some experimental results also suggest a role for ethylene. Our opinion is that ethylene could act as a coordinator of most of the responses to Fe deficiency in Strategy I plants, although it could participate in different ways in different responses, perhaps in combination with other signals such as auxin.

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Chapter 13

TRANSLOCATION OF IRON IN PLANT TISSUES

PETRA BAUER[1] and RÜDIGER HELL[2]

[1]Institute of Plant Genetics and Crop Plant Research, Corrensstr. 3, D-06466 Gatersleben, and Department of Biosciences-Botany, Saarland University, P.O. Box 151150, D-66041 Saarbrücken, Germany. [2]Heidelberg Institute of Plant Sciences, Dept. IV Molecular Biology of Plants, University of Heidelberg, Im Neuenheimer Feld 360, 69120 Heidelberg, Germany. E-mail: rhell@hip.uni-heidelberg.de

Abstract: Iron homeostasis is tightly regulated in plants. To control iron homeostasis processes at the whole plant level, plants employ different components. Here, we review the mechanisms that regulate iron uptake into the root epidermis, long-distance transport of iron within the plant inside the vascular system, iron transport across short distances and intracellular transport of iron between the cytoplasm and intracellular compartments.

Key words: bHLH transcription factor; iron homeostasis; iron transport protein; nicotianamine; yellow stripe-like

1. INTRODUCTION

Typical Fe concentrations in plant tissues range from 10-100 μM. Iron is therefore among the most prevalent micronutrients in plants. Due to its redox properties, Fe is essential for Fe- and heme-dependent enzymes that are involved in diverse physiological processes and for Fe-sulfur clusters in mitochondria and chloroplasts. Free Fe is able to generate toxic hydroxyl radicals in Fenton reactions, which can unspecifically damage cellular components, including DNA and proteins. Iron uptake, distribution and storage are therefore tightly regulated in plants (Hell and Stephan, 2003).

Control of Fe homeostasis is effective at different levels. First, Fe is taken up in a controlled manner from the soil into the root. Due to the low concentrations of bio-available Fe, plants usually need to mobilise Fe in the soil. Iron mobilisation mechanisms are increased when plants experience Fe deficiency. Second, Fe is long-distance transported inside the plant vascular system, for example from root to shoot, or from cotyledons to young shoots.

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Third, Fe is transported at short-distance, intercellularly in the apoplast or intracellularly in the symplast, so that all cells and tissues of a given plant organ are supplied with Fe. Fourth, Fe is transported to the final cellular target destination sites, which can be compartments or biomolecules.

These various Fe translocation processes involve separate Fe transport components, which are summarized here as far as they have been identified.

2. IRON UPTAKE FROM THE SOIL

Upon high Fe supply, organisms tend to use low-affinity Fe transport systems to take up sufficient Fe, while at the same time preventing Fe overload. In contrast, Fe deficiency usually results in the induction of high-affinity Fe transport systems (Andrews *et al.*, 1999; Kaplan, 2002; Van Ho *et al.*, 2002). Depending on the composition and pH of the soil, concentrations of bio-available Fe in the soil can be 10^4 to 10^{15} orders of magnitude lower than Fe concentrations met in plant tissues. Obviously, Fe needs to be mobilised in the soil so that adequate amounts can be taken up. It is not clear at this moment whether plants also possess low- and high-affinity transport systems for Fe. Perhaps their growth in soil habitats requires constitutive, high-affinity Fe transport systems.

Two different strategies for Fe mobilisation have been described for plants (Römheld, 1987). Dicotyledonous and non-graminaceous monocotyledonous plants reduce Fe(III) and take up Fe(II). This so-called Strategy I is accompanied by soil acidification and other physiological and morphological reactions, such as secretion of chelators, root hair proliferation and root transfer cell development (recently reviewed by Curie and Briat, 2003; Hell and Stephan, 2003; Schmidt, 2003). Three essential Strategy I components have been characterized that are induced by Fe deficiency. In the absence of the Fe(III) reductase FRO2 or the Fe(II) transporter IRT1, *Arabidopsis* plants are chlorotic and suffer from Fe deficiency even at normally sufficient Fe supply (Eide *et al.*, 1996; Robinson *et al.*, 1999; Vert *et al.*, 2002). *FRO2* and *IRT1* genes are induced by Fe deficiency in *Arabidopsis* (Eide *et al.*, 1996; Robinson *et al.*, 1999; Vert *et al.*, 2003). Their expression is dependent on the regulator gene *FRU* (= *FER*-like regulator of iron uptake), encoding a basic helix-loop-helix (bHLH)-type transcription factor (Jakoby *et al.*, 2004). In an independent study, this basic helix-loop-helix gene was identified as an iron regulator and named *FIT1* (= Fe-deficiency Induced Transcription Factor 1, Colangelo and Guerinot, 2004). In tomato, a homologous system exists including *LeIRT1*, *LeFRO1* and the bHLH factor LeFER (Bereczky *et al.*, 2003; Li *et al.*, 2004; Ling *et al.*, 2002). It is therefore likely that the Strategy I system is conserved in dicot species.

Grasses depend on an Fe chelation-based Fe mobilisation mechanism, that involves phytosiderophore production and import of Fe(III)-phytosiderophore complexes (Strategy II, reviewed by Mori, 1999). The major genes for phytosiderophore production and a phytosiderophore transporter YS1 (= YELLOW STRIPE1) have been biochemically, genetically or transgenetically analyzed in different grass species [see Curie and Briat (2003), Mori (1999) and reviews in this book for details on the genetic basis for phytosiderophore production; see Curie *et al.* (2001), Schaaf *et al.* (2004) and Roberts *et al.* (2004) for details about maize *YS1*].

Both Strategy I and Strategy II components belong to gene families. The functions of gene family members not involved in root Fe mobilisation are not known. Interestingly, grasses possess homologues of Strategy I genes, and dicot plants contain homologues of some, but not all, Strategy II genes. It is speculated that these additional Strategy I and Strategy II homologues may be involved in Fe transport in organs and tissues other than roots. Such aspects will be further discussed in the following paragraphs.

3. LONG-DISTANCE IRON TRANSPORT

Tracer studies and several plant mutants have provided an overview of the route of Fe from uptake at the root surface to the vascular system and back from leaves to the vasculature to sink organs (reviewed in Hell and Stephan, 2003). Iron allocation seems to be a two-step process with first transport in the xylem followed by remobilization via the phloem. For example, when Fe is supplied directly to the xylem it is first transported to the leaves but not to the apex as the most obvious sink to maintain growth (Zhang *et al.*, 1995). Furthermore, Fe applied to leaves follows the symplastic route and the phloem (Edding and Brown, 1967). This detour is probably caused by incomplete xylem structures in growing organs and their small transpiration rates resulting in little water transport. This has a major impact on plant growth under Fe deficiency, where young leaves suffer more than mature leaves, but also on crop quality, since fruits and storage organs are almost exclusively dependent on Fe import from the phloem. Thus, improving the Fe content of staple food seeds, which is urgently needed in large areas of the world, is a multi-step process difficult to achieve by means of breeding and biotechnology (Grusak and Della Penna, 1999). Unfortunately, our knowledge on the biochemical and molecular processes responsible for the exchange of chelated Fe between these numerous different tissue types is still very fragmentary.

In the xylem, the majority of Fe is complexed as ferric citrate and presumably transported as such. Precise mechanisms for loading of xylem with Fe or ferric citrate and its unloading in leaves have not yet been described.

During germination, the growing shoot apex receives Fe from cotyledons through the phloem, providing a more accessible experimental system for analysis of phloem transport than mature plants. Feeding of ^{55}Fe and ^{65}Zn demonstrated that the vast majority of Fe was associated with the protein fraction in the phloem, while Zn was bound to low-molecular weight compounds. An Fe transport protein (RcITP) was isolated biochemically from *Ricinus communis* cotyledon phloem sap, due to its capability to bind Fe (Krüger *et al.*, 2002). *In vitro*, ITP binds preferentially Fe(III) over Fe(II) but it also forms complexes with Zn, Cu and Mn. One protein molecule is able to bind several metal ions. This was corroborated by the amino acid sequence, which consists mostly of polar residues. Homology searches showed that RcITP belongs to the family of late embryogenesis abundant proteins (LEA) and in particular to the group II called dehydrins (Close, 1996). Dehydrins are highly hydrophilic, stay soluble during heating and are generally expressed during later stages of seed development as well as in other organs in response to drought, cold or high salinity (Nylander *et al.*, 2001). Indeed, a protein with strong sequence similarity to RcITP was found in the *Arabidopsis* genome sequence (AtITP; MIPS code At1g54410). Recombinant AtITP is able to bind Fe(III) and also Ni in comparable ways as RcITP, suggesting an ortholog relationship of the two proteins (C. Krüger, R. Hell, unpublished). Expression patterns of *RcITP* and *AtITP* genes reveal differential expression levels, but nearly ubiquitous presence in tissues with vasculature (Krüger *et al.*, 2002; N. Piening, M. Gahrtz, R. Hell, unpublished).

These findings strongly suggest that ITP as a component of long-distance transport of Fe (and possibly other micronutrients). The conditions that lead to loading of Fe onto ITP in the phloem and unloading at target organs remain unclear. Recently, other members of the dehydrin family have been shown to bind Ca *in vitro*. Interestingly, the Ca binding properties of these proteins are modulated by phosphorylation (Alsheikh *et al.*, 2002; Heyen *et al.*, 2002). It cannot be excluded that this or another post-translational modification mechanism controls loading and unloading of ITP, but chemical conditions such as pH and ion concentration could also affect the number of ions bound per ITP protein.

The metal chelator nicotianamine is transported at long distance in the plant and is able to bind Fe and other metals (Scholz *et al.*, 1992). Nicotianamine plays a key role in intercellular and intracellular transport of Fe that is still not fully elucidated, and may contribute to xylem and phloem

loading and unloading of Fe, as well as long-distance Fe transport (for review see Hell and Stephan, 2003). Nicotianamine is a plant-specific, non-proteinogenic amino acid that derives from the condensation of 3 molecules of S-adenosyl-L-methionine through the action of nicotianamine synthase (Herbik *et al.*, 1999; Higuchi *et al.*, 1999; Ling *et al.*, 1999). Iron deficiency in the nicotianamine-free tomato mutant *chloronerva* results in chlorotic interveinal leaf areas, hence the name of the mutant. Since *chloronerva* plants still have Fe in their leaves, nicotianamine does not seem to be essential for all these processes (Scholz *et al.*, 1992; Takahashi *et al.*, 2003). However, the biochemical observations of preferred binding of Fe(III) by ITP and a more stable complex of Fe(II) with nicotianamine raise the possibility of functional, or even physical, interaction of the protein and the chelator in the phloem, in order to mediate transport or loading processes (Krüger *et al.*, 2002; von Wirén *et al.*, 1999). Another interesting observation related to nicotianamine was that nicotianamine synthase genes from rice were expressed in proximity of vascular tissues, suggesting that indeed these cells may produce nicotianamine for regulating long-distance transport (Inoue *et al.*, 2003). Moreover, *AtYSL2* and *OsYSL2* (*YSL = YELLOWSTRIPE-like*) genes encoding transporter homologs of *ZmYS1* are also expressed in the vascular strands, in proximity to the phloem and xylem (DiDonato *et al.*, 2004; Koike *et al.*, 2004). *AtYSL2* was found induced by Fe supply in roots and leaves and repressed upon Fe deficiency (DiDonato *et al.*, 2004). It was speculated that AtYSL2 may prevent exit of xylem Fe into roots and mature leaves upon Fe deficiency to allow better Fe nutrition of young shoots (DiDonato *et al.*, 2004). Recent studies by Schaaf *et al.* (2005) suggested however that AtYSL2 might not be involved in iron-nicotianamine transport. In contrast, *OsYSL2* was induced by Fe deficiency in leaves and then expressed not only in the vascular strands but in nearly all tissues (Koike *et al.*, 2004). Nicotianamine is also involved in intercellular and intracellular transport and its possible function is discussed further in the following paragraph.

It is an interesting question how Fe loading into and unloading from the vascular system is regulated. The basic helix-loop-helix regulator protein FER is a transcription factor that controls Fe uptake in the root. The *FER* gene is expressed in a developmental pattern along the root. *FER* is not only expressed in the epidermis of the elongation zone, but also in parenchyma cells of the vascular root cylinder in the root hair zone (Ling *et al.*, 2002). *FER* may regulate in these latter parenchymatic cells uptake and transport of Fe. It is also possible; however, that the FER protein delivers or responds to an Fe signal from the shoot. Interestingly, these same parenchymatic cells expressed the Fe transporter genes *LeIRT2* and *LeNRAMP1* (Bereczky *et al.*, 2003). *LeIRT2*, although similar in sequence to *LeIRT1*, is not Fe-regulated

and expressed irrespective of the *FER* genotype (Bereczky *et al.*, 2003; Eckhardt *et al.*, 2001). LeIRT2 may function in a different aspect of metal transport. *LeNRAMP1* expression is partially dependent on *FER* (Bereczky *et al.*, 2003). A homolog of *LeNRAMP1*, *AtNRAMP3*, is also expressed in the vascular cylinder in *Arabidopsis* and may serve the mobilisation of intracellular Fe pools (Thomine *et al*., 2003). *AtFRD3*, encoding a putative oligopeptide transporter of unknown function, is expressed in the root pericycle and vascular cylinder (Green and Rogers, 2004; Rogers and Guerinot, 2002). Chlorotic *frd3* mutant plants over-accumulate Fe in the vascular cylinder of the root (Green and Rogers, 2004). FRD3 therefore seems to act in root xylem loading or in loading of factors for Fe transport towards the leaves.

4. SHORT-DISTANCE IRON TRANSPORT

Within a given plant organ Fe can be transported in the apoplast. Apoplastic Fe is presumably taken up into the cell in a very similar way as soil Fe into the root epidermis. Following uptake from the apoplast at the root surface, the radial transport of Fe to the xylem vessels probably takes place as an Fe(II) complex with nicotianamine, a phytosiderophore-like chelator with high affinity for Fe and several other metals (Stephan *et al.*, 1996). In *Arabidopsis*, it was found that *FRO* and *IRT*-like genes were not only expressed in roots, but also in leaves, where they may be regulated by Fe (Eng *et al.*, 1998; Bauer *et al.*, 2004). The precise functions of these Fe mobilisation-like genes in leaves have not been reported yet. It could be possible that these genes may act in mobilising apoplastic Fe. In addition, Fe can take the symplastic route as demonstrated by feeding studies (Edding and Brown, 1967).

5. INTRACELLULAR IRON TRANSPORT

Inside the cells Fe is translocated to target compartments or target components. It is likely that intracellular organelles express on their membranes their own specific transporters for import or export of Fe. Only few Fe transport proteins have been localised to organellar membranes. AtNRAMP3 protein was found in the vacuolar membrane, where it may serve the export of Fe from vacuoles upon Fe deficiency (Thomine *et al.*, 2003). IDE7, an Fe-regulated ABC-type transporter of unknown function, was also described to be present in the tonoplast (Yamaguchi *et al.*, 2002). Chloroplasts are a major site of Fe function in electron transport chains and

storage of excess Fe bound to ferritin. Iron import into chloroplasts is apparently carried out by uniport-mechanism in a non-specific mechanism, as suggested by successful competition of Fe transport by other micronutrient ions (Shingles *et al.*, 2002). The nicotianamine-free *chloronerva* mutant accumulates Fe precipitates in the chloroplast, but contains no detectable ferritin (Liu *et al.*, 1998; Becker *et al.*, 1995), suggesting that nicotianamine is a prerequisite for transport between chelation in the cytosol and proper storage in target compartments. Mitochondria are another target for Fe, mostly in Fe-sulfur clusters. An *Arabidopsis* mutant lacking a mitochondrial ATP binding cassette transporter shows increased contents of non-heme and non-protein bound Fe, together with increased expression of genes encoding oxygen radical detoxifying enzymes (Kushnir *et al.*, 2001). Frataxin is important for maintenance of mitochondrial Fe levels. Loss-of-function frataxin mutants show altered mitochondrial Fe accumulation and oxidative damage in yeast and humans (known as Friedreich's ataxia) (Babcock *et al.*, 1997; Campuzano *et al.*, 1996). It was proposed that Frataxin binds Fe and might function in mitochondrial Fe-sulfur cluster assembly (Aloria *et al.*, 2004; Bulteau *et al.*, 2004). Frataxin is well conserved in sequence in diverse organisms including plants. AtFH (*Arabidopsis* frataxin) is a functional protein in yeast complementation assays, but its precise function in plants has not been analysed yet (Busi *et al.*, 2004).

6. CONCLUSIONS

Knowledge about translocation of Fe in plants is still rather rudimentary. Most efforts have concentrated on characterizing the genes involved in root Fe uptake systems. Molecular components for Fe transport were identified due to application of biochemical and molecular-genetic techniques. Genome sequence facilitated the discovery of potential Fe uptake homologues of genes that were previously characterized in other organisms. Through availability of genetic and molecular resources these novel plant transport components will be characterized in the next years. It may then be possible to study the function of Fe transport genes in all plant organs, as well as during long and short-distance transport.

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Chapter 14

IRON STRESS RESPONSE AND COMPOSITION OF XYLEM SAP OF STRATEGY II PLANTS

SHIGENAO KAWAI[1] and SHAH ALAM[2]
[1]Faculty of Agriculture, 3-18-8 Ueda, Iwate University, Morioka 020-8550, Japan. E-mail: skawai@iwate-u.ac.jp. [2]Ministry of Agriculture, c/o S.M. Jashim Uddin, 554 Monipur (2nd floor), Mirpur, Dhaka 1216, Bangladesh.

Abstract: A method for the detection of the regions releasing phytosiderophores (PS) in the roots of barley was developed using filter paper and a Fe^{3+} solubilizing assay. The characteristics of the PS release by young roots and root tips are discussed. Feeding experiments with ^{14}C-PS or ^{14}C-PS-Fe^{3+} indicate that both free PS and chelated PS could be absorbed at similar rates by barley roots. It is tentatively suggested that free PS might be absorbed and then subsequently released by roots. The concentrations of PS, organic acids, amino acids, and metal micronutrients were measured in the xylem sap of barley and rice. Phytosiderophore concentrations in the xylem sap of Fe deficient barley were approximately 10- and more than 100-fold higher than those of organic acids and metal micronutrients, respectively. When PS were supplied with Fe^{3+} to barley roots for 4 hours and under hydroponical conditions, the Fe concentrations in xylem sap were enhanced more than 10 times as compared to plants fed with Fe^{3+} only.

Key words: phytosiderophores; reabsorption; release; visualization; xylem sap

1. INTRODUCTION

It is known that Gramineae take up Fe^{3+} by releasing phytosiderophores (PS), solubilizing Fe^{3+} and absorbing the complex of PS-Fe^{3+} by the roots (Marschner, 1995a; Römheld and Marschner, 1986; Römheld, 1987; Takagi, 1976). These plants are known as Strategy II plants. "Phytosiderophores" is a collective name for mugineic acid and its analogues, "the mugineic acid family" or "mugineic acids". The chemical structure and physiological

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function of PS (Sugiura and Nomoto, 1984; Nomoto *et al*., 1987) have been investigated and the outline of the system has been clarified.

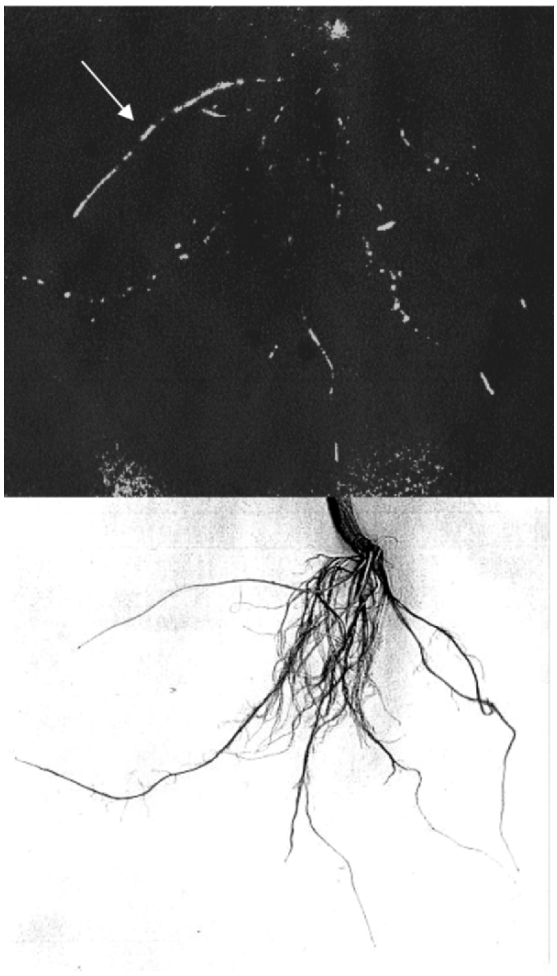

Figure 14-1. Regions of phytosiderophore release by barley roots as detected by Fe solubilizing activity.

However, some details of the system are still unknown. In this chapter, the obtained data related to (1) the regions of release of PS, (2) reabsorption of PS or PS-Fe^{3+} by the roots, and (3) translocation of PS and metal micronutrients to the shoot in grasses, such as barley and rice (Strategy II plants), are reviewed and discussed considering the fate of released PS in grasses.

2. REGIONS FOR RELEASE OF PHYTOSIDEROPHORES BY BARLEY

2.1 Visualization of regions of phytosiderophore release

It has been discussed that PS are released from the young root regions of graminaceous plants grown under Fe deficient (-Fe) conditions (Marschner *et al.*, 1987). The regions for PS release by -Fe barley were visualized in a recent experiment as shown in Figure 14-1 (Yoshida *et al*., 2004). In a similar experiment, it was shown that root tips of Fe sufficient (+Fe) barley released detectable amounts of PS (data not shown). From the results, it is apparent that the activity for PS release varies depending on the roots. It is also noteworthy that a distinct root, indicated by an arrow in the Figure 14-1, showed higher activity for PS release. The distinct root was formed during the -Fe treatment.

2.2 Experimental methods

The methods for visualization of the region of PS release were described elsewhere in detail (Yoshida *et al*., 2004). Barley plants (*Hordeum vulgare* L. cv. Minorimugi) were grown hydroponically in Hoagland and Arnon No.2 medium with or without Fe (Kawai *et al*., 1993). The roots of Fe-deficient barley were put on filter paper and allowed to release PS for 4 hours. The regions of PS release were visualized on the paper by the detection method for PS on TLC described in Kawai *et al.* (1988b).

3. RELEASE AND REABSORPTION OF PHYTOSIDEROPHORES BY BARLEY ROOTS

3.1 Release of ^{14}C-phytosiderophores by barley roots

It has been reported that PS release occurs for 3-4 hours after an increase of the temperature, either in the growth chamber or under natural conditions (Takagi, 1993). In order to confirm the time for PS release and to know the absorption rate of PS or PS-Fe^{3+} in the roots, experiments were conducted using barley grown hydroponically by K. Itoh in Iwate University (personal communication).

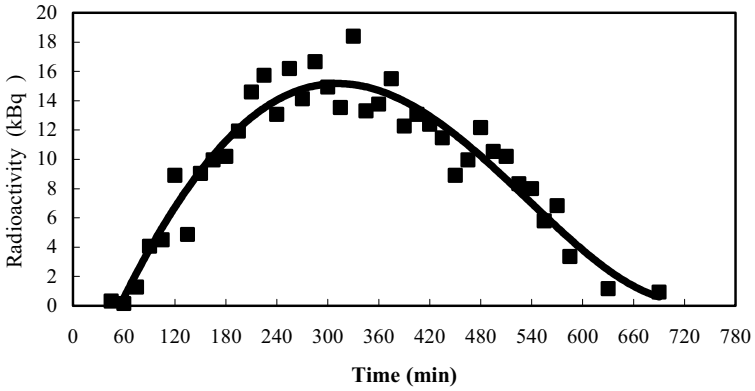

Figure 14-2. Time course of release of ^{14}C-phytosiderophores by barley roots. Roots of -Fe barley plants were fed with ^{14}C-methionine for 1 hour under dark conditions. Plants were then transferred to deionized water and the temperature of the growth chamber was raised from 10ºC to 17ºC. Radioactivity of the root medium was measured.

Figure 14-2 shows the radioactivity of ^{14}C-PS released by barley roots which were transferred to deionized water after raising the temperature of the growth chamber. Released ^{14}C-PS was formed in the roots, which were fed externally with ^{14}C-methionine (Mori and Nishizawa, 1987; Kawai *et al*., 1988a; Kawai *et al.,* 1993). It was shown that ^{14}C-PS release by the roots began after 1 hour and continued for 6 hours after raising the temperature. The fact that PS solely contained the ^{14}C was verified by radio-HPLC.

Almost the entire radioactivity of ^{14}C-PS released into the water was absorbed by the roots within 11 hours in this experiment. Under natural conditions, the time for the absorption of PS may differ, and degradation of PS by microorganisms may be faster than under such experimental conditions (von Wirén *et al*., 1993).

3.2 Absorption of ^{14}C-phytosiderophores or ^{14}C-phytosiderophores-Fe^{3+} complex

It was not known whether free PS is absorbed with a similar rate to that of PS-Fe^{3+} by the roots of grasses. Feeding experiments with ^{14}C-PS and ^{14}C-PS-Fe^{3+} were conducted with -Fe barley roots to solve this question. Our experiments showed that free PS and PS-Fe^{3+} were rapidly absorbed by -Fe barley roots (Figure 14-3 and Figure 14-4). Furthermore, most of the ^{14}C of the fed compounds was absorbed within 2 hours with a similar absorption rate.

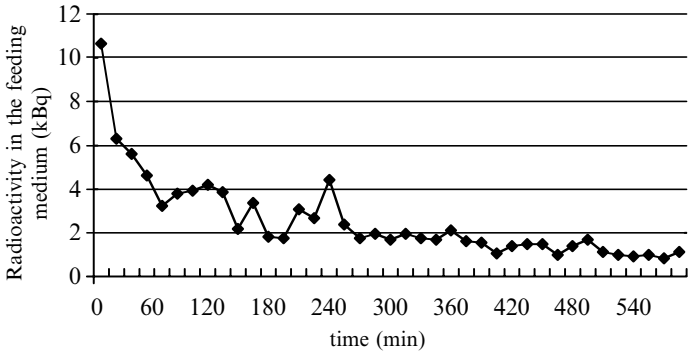

Figure 14-3. Absorption of ^{14}C-phytosiderophores by barley roots. Roots of -Fe barley plants were fed with ^{14}C-PS (20 μM) for 10 hours and radioactivity of the root growing medium was measured.

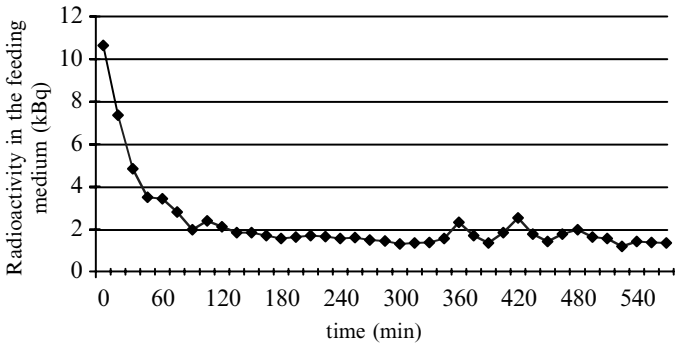

Figure 14-4. Absorption of ^{14}C-phytosiderophores-Fe^{3+} complex by barley roots. Roots of -Fe barley plants were fed with ^{14}C-PS-Fe^{3+} (20 μM) for 10 hours and radioactivity of the root growing medium was measured.

It was observed that the absorption line of ^{14}C-PS fluctuated up to 6 hours (Figure 14-3), differently to what occurs with ^{14}C-PS-Fe^{3+} (Figure 14-4). The difference in the fluctuation in Figure 14-3 and Figure 14-4 could be attributed to the fact that free PS absorbed by the roots may be distinguished from PS-Fe^{3+} in the root cells and may be released from the roots repeatedly during the time for PS release. This will result in a fluctuation of the ^{14}C absorption line. The mechanism for the separation of free PS from PS-Fe^{3+} in the root cell and the recycling of free PS in the rhizosphere has not been explained yet. This phenomenon should be properly studied to clarify the details of Strategy II system in the future.

According to the results of Takagi (1984), -Fe barley plants grown hydroponically release more PS than that needed to meet the Fe demand of the plant. Results indicated that released PS could be absorbed by the roots in the form of both free PS and PS-Fe^{3+}. Absorbed PS-Fe^{3+} must be conveyed to the tissue of vascular bundle through the symplasm (Pitman, 1982). Absorbed free PS may be stored in the root cells and released from the plasmalemma of the root cells the following morning, otherwise free PS will also be translocated to the shoot.

3.3 Experimental methods

Roots of 3 barley plants grown under -Fe conditions were fed with ^{14}C-methionine (185 kBq, 1.8 μM) (Kawai *et al.,* 1993) for 1 hour in the dark and at a temperature of 10°C in a growth chamber. After the feeding of ^{14}C-methionine, the temperature of the growth chamber was raised to 17°C and plants were transferred to a beaker containing 50 ml of deionized water under artificial light of 280 μmol m^{-2} s^{-1} intensity. The radioactivity of the water where the roots were soaked was measured at 15 minute intervals by taking aliquots (0.1 ml) and with a scintillation counter.

Carbon-14 labelled PS for the feeding experiments were obtained as follows: roots of three -Fe barley plants were fed with ^{14}C-methionine (370 kBq, 1.8 μM) at 10°C. Then, plant roots were washed in deionized water, transferred to a beaker containing 50 ml of deionized water and allowed to release PS for 3 hours in an illuminated chamber at 17°C. The root wash containing ^{14}C-PS was condensed in vacuum and diluted with deionized water to 20 ml in a volumetric flask. The concentration of PS in solution was measured by HPLC (Kawai *et al.,* 1988b). In this experiment, the components of PS were confirmed to be mugineic acid and 2'-deoxymugineic acid. Ten ml of the solution of ^{14}C-PS were used for the feeding experiment, and the remaining solution (10 ml) was employed for preparing the ^{14}C-PS-Fe^{3+} complex.

For the preparation of ^{14}C-PS-Fe^{3+} complex, 2 ml of a gelatinous $Fe(OH)_3$ suspension (5 mM) was added to the ^{14}C-PS solution (10 ml). The mixture was kept for 2 hours in an oven at 55°C to facilitate ^{14}C-PS-Fe^{3+} formation, and filtered to remove excess gelatinous $Fe(OH)_3$. The filtered solution was used for the feeding experiment with ^{14}C-PS-Fe^{3+}.

Carbon-14-PS or ^{14}C-PS-Fe^{3+} was dissolved in 50 ml of deionized water and applied to the roots of a -Fe barley plant for 10 hours. The PS concentration of the feeding medium was 20 μM, and pH was adjusted to 7.0. Radioactivity was measured similarly to the experiment of ^{14}C-PS release.

The experiment of ^{14}C-PS release and the feeding experiments of ^{14}C-PS and ^{14}C-PS-Fe^{3+} of this section were started simultaneously when the temperature of the growth chamber was raised to 17°C and the light was on.

4. PHYTOSIDEROPHORES AND METAL TRANSLOCATION IN XYLEM TUBE OF GRAMINEAE

4.1 Content of phytosiderophores, amino acids, organic acids, and metals

The form in which Fe is translocated in Strategy II plants is still a problem that needs to be solved. In dicot plants, citrate is a predominant carrier of Fe in xylem tubes (Brown and Chaney, 1971; Brown and Tiffin, 1965; López-Millán *et al.,* 2000; 2001; Tiffin, 1966). Ion concentrations of xylem exudate from stem incisions have been measured (Hocking, 1980; White *et al.,* 1981). Many researchers have discussed the mechanisms of translocation of micronutrients in monocot or dicot plants (Kochian, 1991; Marschner, 1995b; Tiffin, 1972; Mori, 1998). The function of PS for the translocation of Fe in xylem tubes of grasses, however, has not been well characterized.

It was tentatively concluded by TLC and HPLC analysis that PS do exist in xylem sap of -Fe or +Fe barley plants (Kawai *et al.,* 2001). At present, data are available for the composition of PS, amino acids, organic acids, and metal essential elements in xylem sap of barley plants (Alam *et al.,* 2001b). Xylem sap was collected from decapitated roots of plants grown hydroponically under -Fe or +Fe conditions.

The xylem sap concentrations of macro- or micro-nutrients in -Fe or +Fe barley plants grown hydroponically are shown in Figure 14-5. The -Fe treatment did not affect the concentrations of metal macronutrients such as K, Ca, and Mg. On the other hand, the concentrations of metal micronutrients were affected by -Fe treatment. The Fe concentration decreased, while Mn, Zn, and Cu increased considerably by -Fe treatment.

Many reports have described interactions between micronutrients (Kochian, 1991). For example, the competition between Mn and Fe has been investigated (Heenan and Campbell, 1983; Moraghan, 1979; Somers and Shive, 1942). It is known that Mn toxicity induces Fe deficiency, resulting in PS release in the roots of barley (Alam, 2000). However, the mechanisms leading to increased concentrations of Mn, Zn, and Cu in xylem sap in the – Fe treatment are not clearly understood. One possible reason for this may be the competition between micronutrients on the plasma membrane of root

cells and loading sites of xylem tubes. Further research at the molecular level is required to clarify the mechanism of the competition.

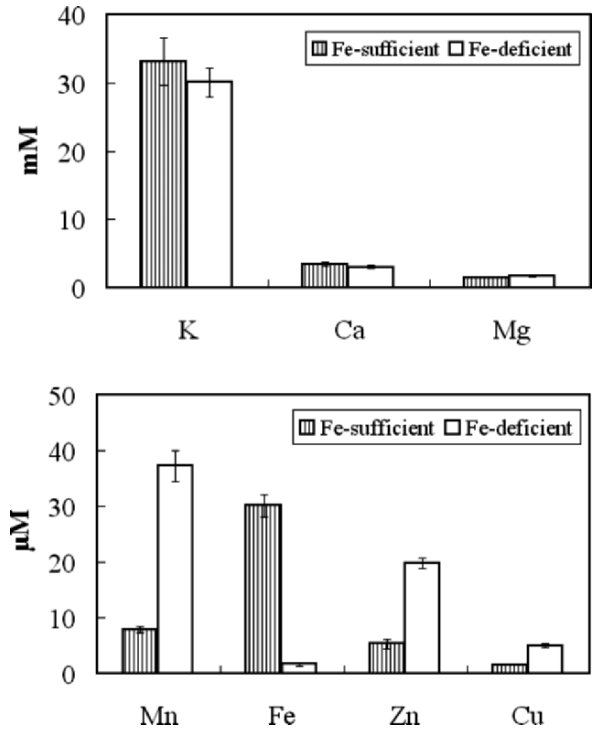

Figure 14-5. Concentrations of some metal essential elements in xylem sap of barley.

Figure 14-6 shows the concentrations of organic acids in xylem sap of barley grown under -Fe or +Fe conditions. It is known that concentrations of organic acids in plant tissues increase under -Fe condition (Iljin, 1951; Abadía *et al.,* 2002). Increased amounts of organic acids in root cells may be passively incorporated into xylem tubes. The citrate concentration, however, was lower than that of Fe in terms of molar concentrations (Figure 14-5 and Figure 14-6), suggesting that citrate might not be a carrier of Fe in the xylem.

The concentrations of PS and amino acids in xylem sap of -Fe and +Fe barley are shown in Table 14-1. As predicted, the PS concentration was much higher in -Fe plants. It was noticeable that the PS concentration was more than 100 times higher than that of Fe in -Fe plants (Kawai *et al.,* 2001). It is considered that a large amount of PS is translocated without chelating Fe^{3+} in xylem tubes, because PS chelate Fe^{3+} with a ratio of 1:1, as suggested

by A. Nagasawa using NMR (personal communication). This also occurs as in the case of PS and Cu (Nomoto *et al.,* 1981). The question arising is why a higher amount of free PS needs to be translocated to the shoot in grasses. Furthermore, the PS concentration was about 20 times higher than that of citrate. The stability constants for the complexes of Fe^{3+} with citrate and PS (mugineic acid) are 11.2 (Brady and Weil, 2002) and 18.1 (Sugiura and Nomoto, 1984), respectively. Murakami *et al.* (1989) estimated the stability constants of PS to be 32.5-33.3. Therefore, based on the result of the experiment and the stability constants, it is inferred that Fe^{3+} may be preferentially chelated with PS in xylem sap of grasses. The dominant form for Fe translocation in xylem sap is a major unresolved problem.

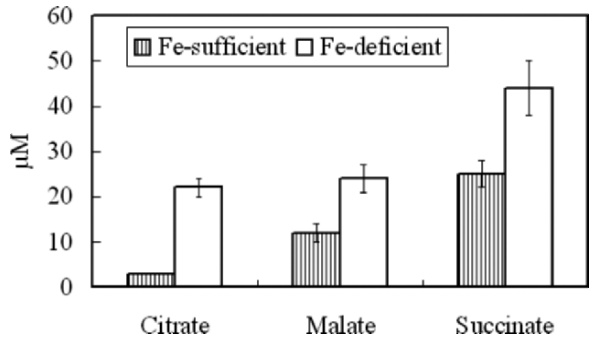

Figure 14-6. Concentration of organic acids in xylem sap of Fe-sufficient or Fe-deficient barley grown hydroponically.

4.2 Concentrations of phytosiderophores and metals of xylem sap of barley and rice grown hydroponically under varied concentrations of iron

As shown in the previous section 4.1, the concentration of PS was much higher than that of Fe in xylem sap of -Fe barley. The ratio of the concentration of PS/Fe varied depending on Fe nutritional status of the plant. In order to obtain data for the PS/Fe ratio as affected by the Fe status of the plant, the xylem sap from grasses grown with various concentrations of Fe (0, 1, 10, 100 μM) in hydroponic culture was collected and analyzed. Iron sources in the hydroponics media for barley and rice were EDTA-Fe (Kawai *et al*., 1993) and citrate-Fe (Alam *et al*., 2003), respectively.

Table 14-1. Concentration of phytosiderophores and amino acids in xylem sap of Fe-sufficient or Fe–deficient barley grown in nutrient solution.

Amino Acid	Fe-sufficient	Fe-deficient
	μM	
Phytosiderophores (PS)	19±2	453±43
Aspartate	129±6	51±2
Threonine	349±36	624±16
Serine	607±13	763±25
Asparagine+Glutamate	1218±100	3868±149
Glutamine	2638±90	1805±95
Cysteine	0.5±0.1	4±1
Proline	32±4	202±32
Glycine	41±5	43±9
Alanine	386±41	380±28
Valine	217±25	433±20
Methionine	6±0.1	28±2
Isoleucine	30±4	33±2
Leucine	26±2	39±4
Tyrosine	20±2	8±1
Phenylalanine	54±2	11±0.3
Total	5771±98	8746±26

Values represent the mean ±SE of 2 replications.

Table 14-2. Amounts and ratios of phytosiderophores and Fe of xylem sap of barley and rice. Xylem sap was collected for 3 hours from 36 plants of barley or 96 plants of rice grown hydroponically in a greenhouse. Iron sources for the cultivation were EDTA-Fe for barley and citrate-Fe for rice.

Barley	PS (nmol plant^{-1} h^{-1})	Fe (nmol plant^{-1} h^{-1})	PS/Fe ratio
Fe-deficient	4.57	0.0122	375
Fe 1 μM	1.65	0.0709	23.3
Fe 10 μM	1.37	0.382	3.59
Fe 100 μM	0.689	0.944	0.730

Rice	PS (pmol plant^{-1} h^{-1})	Fe (pmol plant^{-1} h^{-1})	PS/Fe ratio
Fe-deficient	171	0.774	221
Fe 1 μM	42.6	13.5	3.16
Fe 10 μM	40.1	20.1	2.00
Fe 100 μM	17.4	36.4	0.478

The amount of PS and Fe and the PS/Fe ratios in xylem sap of barley and rice are shown in Table 14-2. The PS/Fe ratio in xylem sap varied depending on the Fe status of the plants. Generally, the PS/Fe ratio decreased according to the increase in Fe concentrations of the medium. Tiffin (1966) reported that citrate/Fe ratios ranged between 1 and 3. The PS/Fe ratios fluctuated in a broader range than those of the citrate/Fe ratio. The PS/Fe ratio could be even less than 1 in the plants grown under Fe abundant condition (100 μM).

Based on the obtained data, it was not possible to conclude that PS were the major carriers for Fe in the xylem of grasses.

The concentrations of metal micronutrients of xylem sap of barley are shown in Figure 14-7. Manganese and Zn concentrations decreased when Fe was added to the medium. However, there was no distinct relationship between the PS concentration and the concentrations of Mn, Zn, and Cu in xylem sap. It seemed that the concentrations of Mn, Zn, and Cu were not related to the concentration of Fe or PS in xylem sap. The function of several chelators for the uptake of metal micronutrients from rhizosphere by the root has been discussed (Linsay 1974; Treeby *et al.,* 1989; Zhang, 1993). The role of PS in plant tissue for the translocation of metal micronutrients also needs further investigation.

4.3 Time course of the concentration of phytosiderophores and iron in xylem sap of barley fed with iron (III)

More data on the relationship between the concentrations of PS and Fe in the xylem sap are necessary to discuss the role of PS on Fe translocation to the shoot in grasses. A feeding experiment with Fe^{3+} was conducted for barley grown hydroponically under -Fe condition. The time course for the changes in the concentrations of PS and Fe was observed after the plants were fed with $FeCl_3$ (30 μM) at 13:00. Eighteen plants of barley were decapitated at 3 hour intervals and xylem sap was collected for 3 hours. The change of the concentration of Fe or PS in xylem sap was monitored after the addition of Fe^{3+}.

As shown in Figure 14-8, the PS concentration in xylem sap decreased after Fe^{3+}supply to the root. The Fe concentration gradually increased with time and PS concentration decreased 7 hours after Fe^{3+}addition. This result clearly showed that the PS/Fe ratio was not constant and it probably varied depending on the accumulation of Fe in root cells. It seemed apparent that concentrations of PS and Fe fluctuated independently.

4.4 Concentration of iron in xylem sap of barley fed with iron(III) and chelators

Takagi (1984) reported that PS could enhance Fe translocation to the shoot more rapidly than EDTA in rice fed with $FeCl_3$ (20 μM). It has been verified that PS are much more effective to enhance the absorption of Fe than the other natural or artificial chelators (Marschner *et al.,* 1986; Römheld and Marschner, 1986). Our preliminary results (Kawai *et al.,* 2001)

reported that PS enhanced Fe concentration in xylem sap more than the synthetic chelator EDTA.

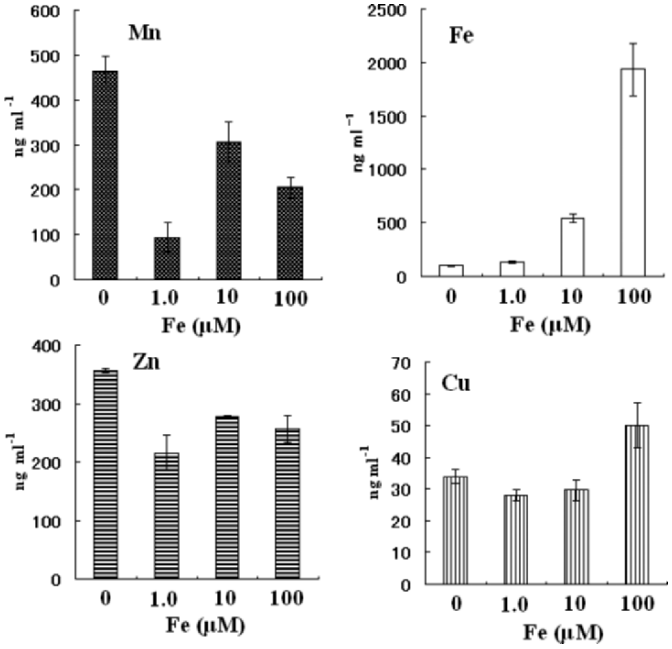

Figure 14-7. Metal concentrations in xylem sap collected from barley grown hydroponically with different concentrations of EDTA-Fe (0, 1, 10, 100 µM).

As a follow-up research, an experiment was conducted to examine whether PS could enhance Fe concentration in xylem sap more than other Fe chelators, when fed to the roots together with Fe^{3+}. Roots of barley plants grown hydroponically under -Fe conditions were supplied with $FeCl_3$ (30 µM) and equimolar amounts of other Fe compounds, such as mugineic acid (PS), deferri-ferrioxamine B (FOB) (a microbial chelator) (van der Helm *et al.*, 1987), EDTA, citrate, and malate from 13:00 to 16:00 p.m., when PS are not released from the roots. At 16:00 p.m., the plants were decapitated and xylem sap was collected for 3 hours.

The concentration of Fe in barley xylem sap was enhanced, with a factor of more than 10, in plants fed with PS and Fe^{3+} as compared to the control plants fed solely with Fe^{3+} (Figure 14-9). This result indicates that the PS-Fe complex was the most effective Fe source as compared with FOB, EDTA, citrate and malate. EDTA was not effective in enhancing Fe concentration in

xylem sap, conversely to what was found in previous studies (Kawai *et al*., 2001).

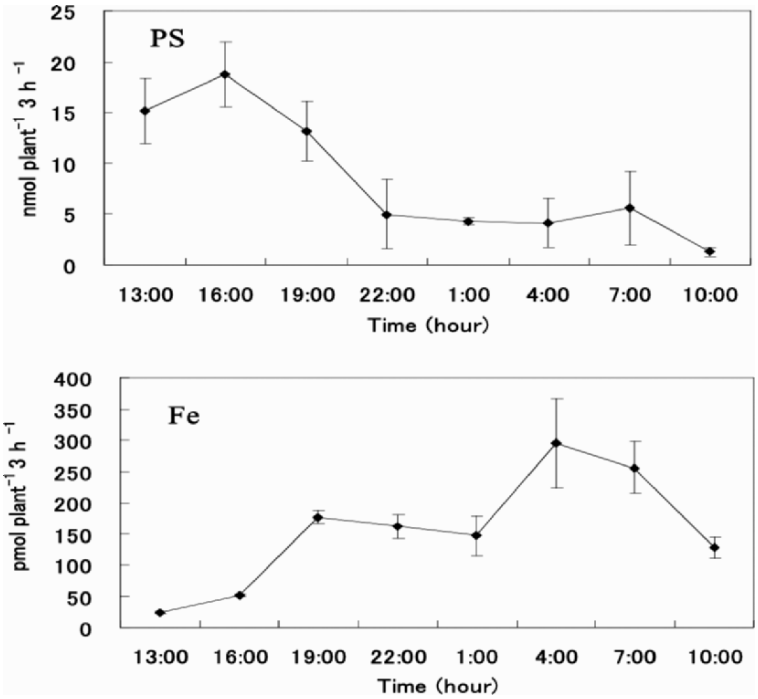

Figure 14-8. Time course of the concentrations of Fe and phytosiderophores in xylem sap of Fe-deficient barley after being fed with $FeCl_3$. Plant roots were fed with 30 µM Fe^{3+} at 13:00 p.m. Plants were decapitated at 13:00, 16:00, 19:00, 22:00, 1:00, 4:00, 7:00, and 10:00 p.m. Xylem sap was collected for 3 hours from the stunts of the plants.

Citrate was the second highest enhancer of the Fe concentration in xylem sap. Citrate has been used as an effective Fe source in nutrient solution by the pioneers of plant nutrition (Hewitt and Smith, 1975). It was also verified in this experiment that citrate-Fe is an adequate Fe source for the plant.

However, the question remains whether the PS-Fe^{3+} absorbed by the roots can be incorporated into the xylem without being dissociated into Fe^{3+} and PS in root cells. Further experiments using ^{59}Fe and ^{14}C isotopes will help us find the answer to this question.

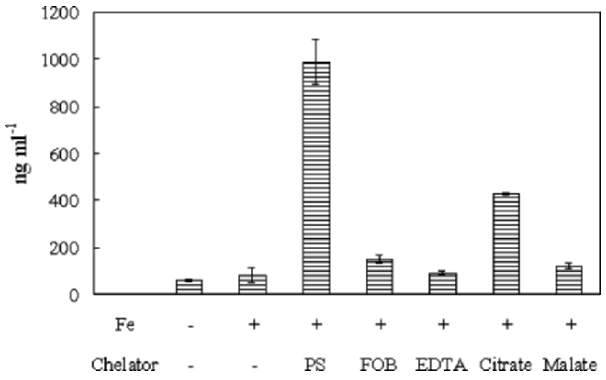

Figure 14-9. Iron concentration of xylem sap collected from Fe deficient barley. Plant roots were treated with 30 μM of various chelators together with an equimolar amount of $FeCl_3$ for 3 hours in the nutrient solution (pH 6.5).

4.5 Diurnal fluctuations of concentrations of phytosiderophores and iron in xylem sap of iron-deficient barley

As described in the previous section, it is known that the PS content of the roots and PS release have diurnal variations (Takagi, 1993) regulated by temperature changes. It is considered that the synthesis of PS occurs in barley roots constantly without any diurnal change (Kawai *et al.,* 1993). The absorption activity of PS-Fe^{3+} was also constant during the day period or the night period, although the absorption activity of PS-Fe^{3+} was larger during the day than during the night (Alam *et al.,* 2004). There was a question that the PS or Fe translocation in xylem sap may have diurnal changes related to a diurnal rhythmicity of PS release.

In the experiment conducted in the greenhouse, 6 bunches (18 plants) of barley plants were decapitated at 3 hour intervals and xylem sap was collected for 3 hours similarly to the experiment described in section 4.3. The amount of PS and Fe was calculated based on the concentration and amount of the xylem sap.

The concentrations of PS and Fe in the xylem sap were fairly constant during 13:00 and 4:00 (Figure 14-10). The amounts, however, showed a peak at 7:00, within the time for the PS release. It seems that the PS and Fe translocation is activated during this time, suggesting that the released and reabsorbed PS might be translocated to the shoot in the morning. The movement of PS might enhance the translocation of Fe to the shoots.

In replicate experiments a similar result was obtained, although the amounts of PS or Fe varied and the timing of the peaks of PS and Fe were not always consistent depending on the experiments. Thus, further experiments under different conditions are necessary to examine whether PS or Fe translocation to the shoot in grasses have diurnal changes.

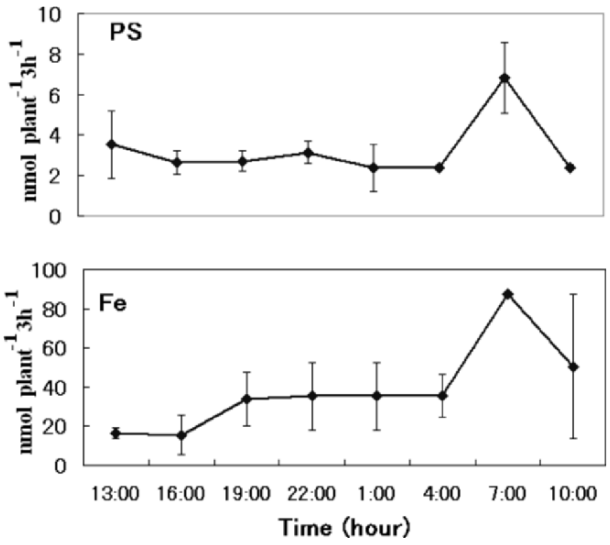

Figure 14-10. Diurnal fluctuations of concentrations of phytosiderophores and iron.

4.6 Effect of root application of various chelators on the translocation of the metals in xylem sap of barley

As indicated in 3.2, it is considered that the absorption of free PS by the root, without Fe, is a usual phenomenon. In addition, feeding with both synthetic and microbial chelators was conducted in section 4.5. Therefore, it was considered that the feeding effect of free chelators on the translocation of metal micronutrients in xylem tubes needs to be examined.

The chelators PS, FOB, and EDTA were dissolved in the hydroponic media (25 μM) and supplied to the root of +Fe or –Fe barley plants for 3 hours in the afternoon. Xylem sap was collected for 3 hours after the supply of chelators (Alam *et al.,* 2001a).

Table 14-3 shows the amount of metal micronutrients translocated in xylem sap. It was apparent that feeding with PS largely enhanced the amount (pmol plant^{-1} h^{-1}) of Fe translocation in xylem tubes of –Fe barley. Feeding with FOB also enhanced Fe translocation slightly. Feeding with EDTA,

however, repressed Fe translocation. Feeding with PS also enhanced the translocation of the other metals. It is speculated that absorbed free PS might chelate other metals in root cells and carry them into the xylem tubes.

It is known that the function of PS for carrying metals from rhizosphere to the cell is specific to Fe^{3+} (Ma *et al.,* 1993) and Zn^{2+} in grasses (von Wirén *et al.,* 1996, Zhang *et al.,* 1991a). It is also known that externally fed PS can solubilize apoplastic Fe of the roots and be absorbed by the roots (Zhang *et al.,* 1991b). However, how the free PS absorbed by the roots function in the root cell has not been discussed. The effect of free PS on the physiological form of the other metals in the root cell needs to be investigated.

Table 14-3. Amounts of phytosiderophores and metals translocated in xylem sap of Fe-deficient barley fed with or without various chelators.

Treatment		PS	Mn	Fe	Zn	Cu
Fe	Chelator	p mol plant^{-1} h^{-1}*				
+	-	502±81	318±29	985±19	219±22	55±3.1
-	-	4820±153	397±31	57±4.5	255±11	61±8.9
-	PS	9871±588	645±38	392±18	392±23	91±11
-	FOB	5561±704	410±44	94±11	253±18	59±9.2
-	EDTA	1939±162	193±12	8.8±2.5	46±4.2	17±2.9

Values were compared with Fe-sufficient plants. This table is a corrected version of the one previously presented in Alam *et al.* (2001a). Values are mean ± SE of 2 replications. *Values previously presented were in nmol plant^{-1} h^{-1}.

4.7 Experimental methods

The methods used in these experiments were described in Kawai *et al*. (2001) and Alam *et al*. (2001a). Barley plants were grown hydroponically in bunches in a greenhouse (Kawai *et al.,* 1988b). Plants were decapitated at about 2 cm above the roots with a stainless steel razor. Xylem sap extruding from the top of the stunts was collected by a capillary tube and kept in a test tube. After collection of the xylem sap, the weight and density of the liquid was measured. The volume of xylem sap was calculated based on weight and density. The xylem sap was stored under –20°C until analysis.

The metal concentrations of xylem sap were determined by graphite furnace atomic absorption spectrophotometry. The components of xylem sap were separated into cationic and neutral + anionic fractions by using cation or anion exchange resin by the method described in Kawai *et al.* (1993). The concentration of PS in the cationic fraction and that of organic acids in the neutral plus anionic fraction were measured by HPLC (Kawai *et al.,* 1988b).

5. DISCUSSION AND FUTURE DIRECTION OF THE RESEARCH

It was confirmed by the experiment shown in section 2.1 that root tips and new (distinct) roots were the main regions for PS release. It is considered that the released PS are absorbed by the young regions of the roots.

It was shown that both free PS and PS-Fe^{3+} complex were absorbed at similar rates by the roots. The absorption rate of free PS had significant fluctuations. It is tentatively suggested that free PS might be absorbed and then subsequently released by roots. It is considered that the phenomenon of PS release involves different mechanisms to be investigated. The process of the evolution of plants generated mechanisms in grasses such as the system for the onset of PS release regulated by temperature or a circadian clock (Takagi, 1993). The plant mechanisms permitting to sense and memorize temperature changes and to follow the circadian rhythms of a day may be one of the most attractive themes in plant physiology.

The function of PS in Fe transport is also a fundamental problem to be solved. In this chapter, root pressure exudation was applied for the measurement of the component of the xylem sap. At present, several new methods have been devised (Schurr, 1999). It has been noted that the concentration of the components in the root pressure exudation may be more condensed than real xylem sap, with the condensation ratio being between 4.3 and 35 (Schurr and Schulze, 1995). Therefore, current data of the concentration of the different components in the xylem sap must be considered carefully. However, the information obtained on the presence of PS or the relative ratios among PS, metals, and organic acids may be considered as reliable.

To the best of our knowledge, the form of Fe in xylem tubes in grasses has not been clarified. Iron might be chelated by PS, citrate, or nicotianamine in the xylem sap of grasses (Rudolph *et al.,* 1985; Scholz *et al.,* 1985). It needs to be mentioned that, at present, data about the concentration of nicotianamine in xylem sap are still scarce.

Furthermore, the unloading step of Fe from the xylem tubes into the shoot needs to be clarified. Minerals must be resorbed from the xylem sap in the shoot (Marschner, 1995b). It seems that the unloading step of Fe or the other metal micronutrients has not been well investigated. Nicotianamine is a probable carrier of Fe and other metals (Pich *et al.,* 1995; Scholz *et al.,* 1988; von Wirén *et al.,* 1999) from the xylem tubes to the interveinal tissues in the shoot. However, the possible role of PS and citrate as Fe^{3+} carriers in the interveinal tissues of grasses cannot be ruled out at present. If Fe^{3+} is carried by PS or citrate in xylem tubes and by nicotianamine in interveinal

tissues, the Fe^{3+} translocated as a Fe^{3+} complex may be reduced to Fe^{2+} at the unloading site, because nicotianamine preferentially chelates Fe^{2+} (von Wirén *et al.,* 1999). The mechanism of Fe reduction in the process of translocation has not been clarified.

In addition to the acropetal transport of Fe, the basipetal translocation of Fe also needs to be investigated. Citrate-Fe is reported as a main form for translocation in the phloem tubes of dicot plants (Maas *et al.,* 1988). It was suggested that phloem sap of rice contained PS (Mori *et al.,* 1991). Nicotianamine was also found to be involved in the translocation of Fe, Cu, Mn, and Zn in phloem tubes in dicot plants (Stephan and Scholz, 1993; Stephan *et al.,* 1995). The behaviour of the putative carriers and metals in plant tissues and the regulation systems in the plants needs to be investigated, especially in Poaceae and other food crops.

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Chapter 15

THE ROLE OF ZIP FAMILY MEMBERS IN IRON TRANSPORT

BRENDA PARSON HALL and MARY LOU GUERINOT
Department of Biological Sciences, Dartmouth College, Hanover, New Hampshire 03755, USA. E-mail: guerinot@darmouth.edu

Abstract: Deficiencies of micronutrients such as iron negatively impact plant and human health. Thus, understanding the uptake of iron from the soil is clearly the first step towards engineering plants better able to grow in soils now considered marginal, to increase crop biomass on soils now in cultivation and to improve the iron content of plant-based foods. IRT1 is the major iron transporter induced in the roots during the iron deficiency response. It is a founding member of the ZIP family of metal transporters, which includes over 100 proteins found in many diverse organisms including bacteria, fungi, mammals and plants. Here we review the role of IRT1 and other ZIP family members in iron transport in a variety of plant species including tomato, rice, *Medicago truncatula* and the hyperaccumulator *Thlaspi caerulescens*. Using IRT1 as the example, we then review how iron controls gene expression, transcriptionally through iron regulated transcription factors such as FER and FIT1 and post-transcriptionally, probably via protein turnover. Ultimately, we hope to understand iron transport at the whole plant level; progress on determining how plants sense and respond to iron is also presented.

Key words: iron homeostasis; iron transport; IRT1; metal; transporters; ZIP

1. INTRODUCTION

Iron is required for various essential life processes including DNA synthesis, oxygen transport and photosynthesis. In this context, iron is extremely versatile due to its reversible redox potentials that allow it to form octahedral complexes with different ligands. In particular, iron functions as an important cofactor in metalloenzymes, acting as an electron donor and electron acceptor. Despite its abundance in most soils, iron is the third most

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limiting nutrient for plant growth due to its propensity to form stable, insoluble oxyhydroxides that effectively limit free Fe(III) to an equilibrium concentration of 10^{-17} M at neutral pH, a value far below that required for the optimal growth of plants (Guerinot and Yi, 1994). If the mechanisms of iron uptake, distribution and regulation in plants were clearly understood, it might be feasible to engineer plants that are better able to grow under iron limiting conditions. The study of iron homeostasis in plants also has implications for human health and nutrition, as iron deficiency is a major problem wherever plant-based diets are common. Indeed, iron deficiency is the leading human nutritional disorder in the world today, affecting over 3 billion people (WHO, 2005).

When faced with iron deficiency, Strategy I plants, which include all plants except the grasses, have three main responses (as reviewed in Curie and Briat, 2003; Hell and Stephan, 2003). They release protons into the surrounding rhizosphere via a proton-ATPase. This serves to lower the pH, thereby increasing the amount of free, soluble Fe(III). Additionally, a plasma membrane-bound Fe(III) chelate reductase is induced which reduces Fe(III) to the more soluble Fe(II) form. Finally, the Fe(II) is taken in via an inducible Fe(II) transporter. The grasses, which include a number of the world's major crops such as corn, wheat and rice, use a chelation strategy (referred to as Strategy II) to obtain iron from the soil. Low molecular weight compounds, known as the mugineic acid (MA) family of phytosiderophores, are released into the soil where they bind soluble Fe(III). The phytosiderophore-Fe(III) complexes can then be transported into the plant.

Here we will focus on what we have learned about a family of metal transporters, the ZIP family, and the role its members play in iron transport in plants. One of the ZIP family members, IRT1, is the major iron transporter induced in the Strategy I response (Vert *et al.*, 2002). It is also likely that ZIP family members have a role to play in iron transport in Strategy II plants as well.

2. THE ZIP FAMILY

The ZIP family was named after its founding members, Zrt1p and Zrt2p in yeast and IRT1 in *A. thaliana* (Eide *et al.*, 1996; Zhao and Eide, 1996a, 1996b). As we will discuss below, IRT1 is the major iron transporter responsible for iron uptake from the soil and Zrt1p and Zrt2p are zinc transporters. There are now more than 100 members of this family found in many diverse organisms including bacteria (Grass *et al.*, 2002), *Drosophila* (Mathews *et al.*, 2005), mammals (Dufner-Beattie *et al.*, 2003; Eide, 2003;

Taylor and Nicholson, 2003) and zebrafish (Yamashita *et al.*, 2004). Various members of the ZIP family have been shown to transport iron, manganese, zinc, copper and cadmium. All ZIP proteins are predicted to be integral membrane proteins; most family members are predicted to have eight transmembrane domains with a variable region between transmembrane domains III and IV (Eng *et al.*, 1998; Guerinot, 2000). The variable region is often histidine-rich and has been shown to localize to the cytoplasm in yeast (Gitan and Eide, 2000) and in humans (Gaither and Eide, 2000; Gaither and Eide, 2001). Where characterized, ZIP proteins have been found to transport metal ions from the cell exterior or lumen of intracellular organelles into the cytoplasm. The sole exception is GmZIP1 which has been localized to the peribacteroid membrane of soybean nodules (Moreau *et al.*, 2002). Antibodies to GmZIP1 inhibited zinc uptake by symbiosomes, suggesting that this protein is responsible for transporting metal ions from the cytosol into the symbiosome. However, GmZIP1 was also shown to rescue the zinc uptake mutant of yeast i.e. it is capable of transporting zinc into the cytosol. If GmZIP1 has the same orientation in both the peribacteroid and yeast membranes, then it must be capable of bidirectional transport.

The ZIP family has been divided into four sub-groups based on amino acid sequence similarity: Subfamily I, Subfamily II, LIV-1, and gufA (gene of unknown function) (Gaither and Eide, 2001; Taylor *et al.*, 2003). The LIV-1 subfamily is named after its founder member, an estrogen-regulated gene involved in the formation of breast cancer. This sub-family has been recently re-named LZT, for LIV-1 subfamily of ZIP zinc Transporters (Taylor *et al.*, 2003). LZT proteins are similar to other ZIP proteins with respect to predicted secondary structure but they have a unique motif (HEXPHEXGD) in transmembrane V. Embedded within this motif is the consensus sequence of the catalytic zinc binding site of metalloproteases (HEXXH). This domain had been previously recognized in some presumptive ZIP family members (Begum *et al.*, 2002; Suzuki and Endo, 2002), with Suzuki and Endo (2002) referring to it as the HELP domain. The LZT sub-family includes members from at least 12 different species including humans, mouse, drosophila, plants, yeast and bacteria. The one characterized member of the LZT family from *Arabidopsis*, IAR1, has been localized to the ER and may play a role in zinc homeostasis (Lasswell *et al.*, 2000). IAR1 was originally identified as a mutant that was resistant to the inhibitory effects of several IAA-amino acid conjugates.

Arabidopsis is predicted to have 15 *ZIP* genes which belong to subfamily I (Mäser *et al.*, 2001). One obvious question to ask is why does *Arabidopsis* need so many ZIP transporters? We know that metals are transported from the soil into the root and then must cross both cellular and organellar membranes as they are distributed throughout the plant. Thus, we might

expect different ZIP transporters to be functioning in different tissues. We can also expect that different members might function at different membranes; we have now localized various ZIP family members to the plasma membrane, to endocytotic vesicles and to the ER. In addition to functioning at different locations, some of the ZIP transporters may have different substrate specificities as well as different affinities for metals: high-affinity systems that are active in metal limiting conditions and low-affinity systems that function when substrates are more abundant. In yeast, ZIP family Zrt1p is a high affinity zinc transporter whereas ZIP family member Zrt2p is a low affinity zinc transporter (Zhao and Eide, 1996a, 1996b). Various molecular approaches ultimately can tell us not only in what tissue and cell type certain transporters are expressed but also where within a cell each is expressed. We have also identified plant mutants carrying insertions in all of the *Arabidopsis ZIP* transporter genes; this will greatly help in assigning functions. Having cloned genes in hand is also allowing us to undertake structure–function studies on the encoded proteins themselves (Rogers *et al.*, 2000). Finally, moving beyond how any one transporter functions, we need to keep in mind that we want to understand metal transport at the whole plant level and to use such knowledge to develop plants with enhanced mineral content as well as plants that bioaccumulate or exclude potentially toxic cations. Such understanding will require knowledge of how metal levels are sensed by plants and how metals control gene expression.

3. IRT1

IRT1 was originally identified by its ability to rescue an iron uptake mutant of yeast (Eide *et al.*, 1996). Consistent with this, IRT1 is predominantly expressed in the roots of iron deficient plants. We now know from complementation and uptake studies in yeast that IRT1 is able to transport manganese, zinc and cadmium in addition to iron (Korshunova *et al.*, 1999; Rogers *et al.*, 2000). The broad cation range of IRT1 makes it an excellent model system for the study of residues involved in metal recognition and transport. To determine the importance of individual amino acid side chains for metal transport, we substituted alanine for a number of amino acid residues that are predicted to be potential metal ligands and that are highly conserved among ZIP family members (Rogers *et al.*, 2000). Replacement of a glutamic acid residue with alanine at position 103 increases the substrate specificity of the transporter by selectively eliminating its ability to transport zinc. Two other mutations, replacing the aspartic acid residues at either position 100 or 136 with alanine, also increase the metal

selectivity of IRT1 by eliminating transport of both iron and manganese. A number of other conserved residues in or near transmembrane domains (H97, H197, H224 and E228) are essential for all transport function. It is clear from this study that we can alter the metal transport profile of IRT1. Such ability could prove beneficial in preventing iron deficient plants from accumulating unwanted metals (see below).

3.1 *irt1* mutants

Three groups independently identified *irt1* mutants. We used a reverse genetic screen to identify a line carrying a T-DNA insertion in the third exon of *IRT1* (Vert *et al.*, 2002). This *irt1* mutant line is severely impaired in iron uptake and dies before setting seed unless the plants are watered with high levels of iron, demonstrating that *IRT1* is an essential gene. Unlike wild type plants, *irt1* mutant plants did not accumulate cobalt, manganese or zinc when grown under iron deficiency. *irt1* mutants also did not accumulate cadmium; *irt1* roots contained five times less cadmium than wild type roots. Thus, IRT1 appears to be responsible for the increases in other divalent cations such as cadmium, manganese and zinc that have previously been reported in iron deficient plants (Cohen *et al.*, 1998a; Welch *et al.*, 1993). Henriques *et al.* (2002) screened a different collection of T-DNA mutants and found a line with an insertion 133 bp upstream of the translational start of *IRT1*. They also reported that inactivation of *IRT1* leads to lethality. They characterized a number of cell differentiation defects in their *irt1* mutant, including a reduction of chloroplast thylakoid stacking into grana, reduced number of vascular bundles in stems and irregular patterns of enlarged endodermal and cortex cells in the roots. The third group to identify an *irt1* mutant was carrying out a screen for alterations in the effective quantum yield of photosystem II (Varotto *et al.*, 2002). *irt1* mutants exhibited a drastic decrease in the abundance of proteins of the photosynthetic apparatus and of chlorophyll, which has a marked effect on photosynthetic efficiency.

Because the *irt1* mutation is lethal, it is clear that no other *ZIP* gene can substitute for its loss. Even overexpression of *IRT2*, the closest homolog of *IRT1*, cannot compensate for loss of *IRT1* (Varotto *et al.*, 2002; Vert *et al.*, 2002). Addition of excess iron (but not cobalt, manganese or zinc) can overcome the loss of *IRT1*, suggesting that IRT1 is likely to function in iron uptake from the soil. IRT1 is strongly upregulated in the roots of iron starved plants but it is also fairly highly expressed in flowers. It is still not clear what the contribution of IRT1 may be in flowers because *irt1* mutants usually die before forming flowers and setting seed. It would be interesting to express IRT1 under the control of a root-specific promoter and then examine the phenotype caused by lack of expression of IRT1 in flowers.

3.2 IRT1 orthologs

There are now many presumptive orthologs of IRT1 in the database but very few of these have been studied experimentally. We will briefly summarize the published observations on presumptive IRT1 orthologs.

3.2.1 Tomato

Using *AtIRT1* as a probe, two tomato genes, *LeIRT1* and *LeIRT2*, were identified that encoded proteins 64% and 62% identical to AtIRT1, respectively (Eckhardt *et al.*, 2001). Both LeIRT1 and LeIRT2 can rescue the iron uptake mutant of yeast but only LeIRT1 is iron regulated. One obvious question is whether these two genes are the orthologs of the *Arabidopsis IRT1* and *IRT2* genes. Whereas the tomato genes are arranged in tandem in a tail-to-tail configuration, the *Arabidopsis* genes are arranged in head-to-tail orientation, suggesting that independent duplication events have taken place in the two genomes. Furthermore, LeIRT1 and LeIRT2 are very similar in sequence whereas AtIRT1 and AtIRT2 are more diverged, suggesting that the duplication event took place earlier in the *Arabidopsis* genome than in the tomato genome. Bauer *et al.* (Bauer *et al.*, 2004) have attempted to deduce functional gene pairs from *Arabidopsis* and tomato using sequence information and map position as well as gene expression patterns. They conclude that LeIRT1 is probably the ortholog of AtIRT1 but that LeIRT2 is not orthologous to AtIRT2. Five of the fifteen *Arabidopsis* ZIP proteins cluster with LeIRT1 and LeIRT2: AtIRT1, AtIRT2, AtZIP7, AtZIP8 and AtZIP10; according to their analysis, LeIRT1 and LeIRT2 are most similar to AtZIP10. *AtZIP10* expression is very low and is not up-regulated under iron deficiency (Guerinot, unpublished data and expression data available at http://www.cbs.umn.edu/*Arabidopsis*/ and http://mpss.udel.edu/at/java.html).

3.2.2 Pea

A pea isolog of IRT1, RIT1, is 79% similar and 63% identical to AtIRT1. When expressed in yeast, RIT1 can complement an iron transport deficient yeast strain (*fet3fet4*), a zinc transport deficient yeast strain (*zrt1zrt2*), and a manganese deficient yeast strain (*smf1*) (Cohen *et al.*, 1998b, 2004). Using radiotracer techniques, RIT1 has been shown to mediate high affinity iron uptake and low affinity zinc and cadmium uptake in yeast (Cohen *et al.*, 2004). Like *IRT1*, *RIT1* is expressed in roots in response to iron deficiency.

3.2.3 Medicago

Three of the six *ZIP* genes identified to date from the model legume *Medicago truncatula* were able to rescue the iron uptake mutant of yeast (López-Millán *et al.*, 2004). One of these, MtZIP6, groups with the other ZIP transporters known to transport iron, namely AtIRT1, AtIRT2, RIT1, LeIRT1 and LeIRT2. *MtZIP6* is expressed in both roots and leaves, with higher levels of expression detected in leaves. Its expression does not appear to change in response to alterations in metal levels (various levels of manganese, zinc and iron were tested). The fact that *MtZIP6* is more highly expressed in leaves and is not iron regulated makes it quite distinct from AtIRT1.

3.2.4 Rice

There are at least 13 ZIP proteins in rice, two of which cluster with *Arabidopsis* IRT1 and IRT2 (Gross *et al.*, 2003); one of these is OsIRT1 and the other gene has been named OsIRT2. OsIRT1 is predominantly expressed in roots and is induced under iron as well as copper deficiency (Bughio *et al.*, 2002). OsIRT1, however, appears to only transport iron and not copper, based on its ability to rescue an iron uptake mutant of yeast but not a copper uptake mutant of yeast. These data suggest that at least some of the grasses may use both Strategy I and Strategy II to take up iron. As Fe(II) would be the predominant form of iron available when rice is grown under submerged conditions, it makes sense that rice would have Fe(II) transporters in addition to Fe(III) transporters.

3.2.5 Hyperaccumulators

Over 400 metal hyperaccumulating species of plants have been reported, of which about 16 are zinc hyperaccumulators (containing more than 10,000 μg Zn g^{-1} in shoot dry matter) (Brooks *et al.*, 1998). Amazingly, certain populations of *Thlaspi caerulescens* can tolerate up to 40,000 μg Zn g^{-1} tissue in their shoots whereas normal Zn concentration for most plants is between 20 and 100 μg g^{-1} tissue. There is great interest in hyperaccumulators because of their potential for use in extracting metals from soils, either in phytoremediation (Salt *et al.*, 1998) or phytomining (Brooks *et al.*, 1998). Several *Arabidopsis* sub-family I members have been implicated in the zinc hyperaccumulating response (Becher *et al.*, 2004; Burleigh *et al.*, 2003; Lasat *et al.*, 1999; Lombi *et al.*, 2002; Pence *et al.*, 2000; Weber *et al.*, 2004).

In addition to accumulating zinc, the Ganges ecotype of *T. caerulescens* is able to accumulate up to 10,000 mg Cd Kg^{-1} in its shoot dry matter without suffering toxicity (Lombi *et al.*, 2000). Because IRT1 is known to transport cadmium, Lombi *et al.* (2002) examined the effect of iron deficiency on the ability of the Ganges ecotype to accumulate cadmium. Cadmium uptake was significantly enhanced by iron deficiency in this ecotype but not in an ecotype (Prayon) that did not accumulate high levels of cadmium (Lombi *et al.*, 2002). Furthermore, the steady state level of *TcIRT1* mRNA was increased in the roots of the Ganges ecotype under iron deficiency but not in the roots of the Prayon ecotype. Consistent with the idea that TcIRT1 might be involved in cadmium uptake, Roosens *et al.* (2004) have shown that iron levels are lower in the Ganges ecotype but not in other ecotypes when the plants are grown with cadmium.

3.3 Control of IRT1 expression

Examination of the effect of iron on the steady state levels of *IRT1* mRNA demonstrated that *IRT1* was induced upon iron removal and repressed upon iron resupply (Connolly *et al.*, 2002). These changes in transcript levels could be detected within 24 hours of transferring plants to or from iron-deficient growth conditions. *FRO2*, the gene that encodes the root Fe(III) chelate reductase, shows an identical pattern of mRNA accumulation as *IRT1,* suggesting that these genes may be part of the same iron regulon (Connolly *et al.*, 2003). To our surprise, we have recently identified a bHLH transcription factor, FIT1, that differentially regulates these two genes (Colangelo and Guerinot, 2004). In a *fit1* loss of function mutant, no *FRO2* mRNA could be detected but *IRT1* mRNA was still present and still showed iron regulation, although levels were reduced relative to wild type. Most interestingly, despite the presence of *IRT1* mRNA, no IRT1 protein could be detected. This result suggests that turnover of IRT1 protein is a FIT1-dependent process. Previous studies on overexpression of IRT1 in *A. thaliana* had already revealed post-transcriptional control of IRT1 (Connolly *et al.*, 2002). Plants overexpressing *IRT1* accumulated mRNA throughout the plant, yet IRT1 protein could only be detected in the roots of iron deficient plants. It is likely that the IRT1 protein is being controlled by ubiquitination followed by endocytosis and proteolysis. ZRT1, a yeast ZIP family member, is subject to ubiquitin-mediated protein turnover that is dependent on a critical lysine residue in the variable loop region (Gitan and Eide, 2000). IRT1 has two lysine residues in the analogous region that could serve as ubiquitination sites to mediate protein degradation. Interestingly, in contrast to Zrt1p which is degraded in response to zinc, a number of the mammalian ZIP proteins are still detected in the presence of zinc (Kim *et al.*, 2004;

Wang *et al.*, 2004a). It is their localization which changes in response to zinc status. Under zinc replete conditions, mZIP1, mZIP3, mZIP4 and hZIP4 are found in intracellular vesicles. When cells are depleted for zinc, these proteins accumulate at the plasma membrane. Such metal dependent trafficking may be general feature of metal homeostasis; we know that copper also regulates trafficking of copper transporters (Lutsenko and Petris, 2003). It will be interesting to see if common regulatory mechanisms underlie the trafficking of all the various metal transporters.

We originally identified *FIT1* in a microarray experiment as a gene whose message was iron regulated in wild type roots but deregulated in roots of the *frd3* mutant (Colangelo and Guerinot, 2004). Jakoby *et al.* (2004) identified the same gene, which they named *FRU* (FER-like regulator of iron uptake), because of its similarity to the tomato *fer* gene. The *fer* tomato mutant was initially characterized as being unable to induce the Strategy I response (Brown *et al.*, 1971; Brown and Ambler, 1974). The *fer* gene was positionally cloned and was shown to encode a root specific bHLH protein (Ling *et al.*, 2002). Although FER and FIT1(FRU) appear to be orthologs, there are some differences in their expression patterns. *Fer* expression was originally reported to be independent of iron supply in tomato (Ling *et al.*, 2002); however, a more recent report does show downregulation of *FER* when iron is supplied at 100 μM (Brumbarova and Bauer, 2005). *FER* message is not detectable in the epidermis of the mature root hair zone (Ling *et al.*, 2002), whereas *FIT*(*FRU*) expression can be seen by GUS staining in the differentiation zone in *Arabidopsis* (Colangelo and Guerinot, 2004). Another difference between *fer* in tomato and *FIT1* (*FRU*) in *Arabidopsis* concerns the effect of overexpressing these proteins. When *fer* was overexpressed in tomato, it did not lead to overepression of LeIRT1 (Bereczky *et al.*, 2003; Brumbarova and Bauer, 2005). Overexpression of FIT1 also did not lead to up-regulation of IRT1 or FRO2 (Colangelo and Guerinot, 2004). However, Jakoby *et al.* (2004) report that overexpression of FRU did result in some changes in the expression of IRT1 and FRO2.

One big question in the iron field concerns the nature and location of the iron sensor(s) in the plant. Split root experiments have shown that expression of *IRT1* is controlled at two levels (Vert *et al.*, 2003). First, there is local control via the root iron pool and secondly, there is systemic control involving a shoot-borne signal. Reciprocal grafting experiments with two pea mutants had previously suggested the presence of a shoot-derived signal that regulates the root response to iron starvation (Grusak and Pezeshgi, 1996). *dgl* (degenerative leaves) and *brz* (bronze) mutants have constitutive iron uptake responses despite having high levels of iron in their shoots (Grusak *et al.*, 1990; Grusak and Pezeshgi, 1996). When the shoots of either the *dgl* or *brz* mutant were grafted onto wild type roots, the plants showed

elevated Fe(III) chelate reductase activity. This elevated activity was not seen when the wild type shoots were grafted onto mutant roots, indicating there is a signal that travels from the shoot to the root in response to a surplus or deficiency in iron (Grusak and Pezeshgi, 1996). While the specifics of the iron sensing pathway are still not known, it is thought that the signal is most likely transmitted through the phloem (Maas *et al.*, 1988). The *Arabidopsis* split root experiments also suggested that some iron must be present in order to induce expression of *IRT1* and *FRO2* (Vert *et al.*, 2003). While it is understandable that plants exposed to iron deficiency would benefit by expressing *IRT1* and *FRO2* at high levels when iron is present for uptake, there must be some threshold after which post-transcriptional regulation of these genes would kick in. Experiments with AtNRAMP3, a vacuolar iron transporter that is proposed to pump iron from the vacuole into the cytosol, lend support to the idea that *IRT1* and *FRO2* respond to cellular levels of iron (Thomine *et al.*, 2003). Overexpression of AtNRAMP3 downregulates the expression of both *IRT1* and *FRO2*, presumably by increasing cytosolic iron levels.

In the tomato mutant *chloronerva*, steady state levels of *LeIRT1* mRNA are high regardless of iron levels (Bereczky *et al.*, 2003). This mutant constitutively expresses all of its iron uptake responses and this phenotype can be reversed by exogenous application of nicotianamine (Stephan and Grün, 1989). The *chloronerva* gene encodes nicotianamine synthase which is responsible for the synthesis of nicotianamine from S-adenosyl methionine (Ling *et al.*, 1999). Nicotianamine is thought to function as an intracellular iron chelator and in the transport of iron between cells and in the phloem (Hider *et al.*, 2004). The fact that *IRT1* mRNA levels are high in the *chloronerva* mutant certainly suggests that nicotianamine is required for the down regulation of LeIRT1 usually seen under iron sufficiency (Bereczky *et al.*, 2003).

Arabidopsis halleri is well known for its metal tolerance and hyperaccumulation. It is one of 16 recognized zinc hyperaccumulators capable of accumulating zinc in its tissues to concentrations of greater than 1% of its dry matter. It is found throughout Europe on both contaminated and uncontaminated sites. DNA sequences from *A. halleri* are on average 94% identical to those of *A. thaliana*. This has allowed interspecies comparisons to investigate the molecular basis and mechanisms of metal hyperaccumulation. Perhaps not surprisingly, the two species respond quite distinctly to increased levels of zinc (Becher *et al.*, 2004; Weber *et al.*, 2004). Of interest here is that transcript levels of *IRT1* were dramatically increased in *A. thaliana* after 4 days of exposure to high zinc (100 μM) (Becher *et al.*, 2004). This correlated with an increase in transcript levels of *FRO2* and ferric chelate reductase activity. Connolly *et al.* (2002) had also

reported an increase in *IRT1* transcript levels when iron deficient plants were exposed to zinc excess for 3 days. However, IRT1 protein was not detectable. *IRT1* is not thought to be involved in hyperaccumulation in *A. halleri*. Other *ZIP* genes, in particular *ZIP6* and *ZIP9*, are very highly expressed in *A. halleri* relative to *A. thaliana.*

4. IRT2

IRT1 and *IRT2* are arranged in tandem on Chromosome 4. The proteins are 69% identical and 82% similar; their chromosomal arrangement suggests that they arose via gene duplication. *A priori*, one might speculate that these two genes have redundant functions. Both genes are expressed in the outer layers of the root and steady state mRNA levels for both genes increase under iron deficiency, although the expression of *IRT2* is several fold lower than *IRT1* (Vert *et al.*, 2001; Vert *et al.*, 2002). Like IRT1, IRT2 is able to restore growth to an iron uptake mutant of yeast and a zinc uptake mutant of yeast (Vert *et al.*, 2001). However, unlike IRT1, IRT2 does not complement a yeast manganese uptake mutant or cause sensitivity to cadmium when expressed in wild type yeast (Vert *et al.*, 2001). These differences might indicate that IRT1 and IRT2 have different affinities for these metals but since these data are from heterologous expression, they could also reflect differences in expression in yeast. The best evidence that IRT1 and IRT2 are not redundant comes from the analysis of loss of function mutants. As mentioned above, *IRT2* is unable to complement the seedling lethality phenotype of the *irt1* mutant even when expressed from the strong 35S promoter (Varotto *et al.*, 2002; Vert *et al.*, 2002). Furthermore, an *irt2* mutant itself does not appear to have any phenotype when grown under low iron conditions (Varotto *et al.*, 2002; Vert *et al.*, 2001).

5. ZIP1

AtZIP1 was originally identified due to its ability to rescue a zinc uptake mutant of yeast (Grotz *et al.*, 1998). It is mainly expressed in the roots of zinc deficient plants and its expression is not regulated in response to iron deficiency. AtZIP1 has recently been reported to rescue an *E. coli* strain carrying disruptions in all known iron uptake systems, suggesting that AtZIP1can transport iron (Grass *et al.*, 2005). This is interesting in light of the fact that ZIP1 cannot rescue a yeast iron uptake mutant (Guerinot, 2000). There is one other study implicating AtZIP1 in iron transport. When AtZIP was overexpressed in barley under the control of the 35S promoter, seeds

from soil grown transgenic lines had higher zinc and iron levels than control plants (Ramesh *et al.*, 2004). The seeds were smaller but the overall zinc and iron content per seed was 1.5-2 times higher in the transgenic plants than in controls.

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Chapter 16

ROLE OF *FRD3* IN IRON TRANSLOCATION AND HOMEOSTASIS

ELIZABETH E. ROGERS
Departments of Biochemistry and Nutritional Sciences at the University of Missouri, Columbia, MO 65211, USA. E-mail: rogersee@missouri.edu

Abstract: Relatively little is known about the process of root to shoot iron translocation. This chapter discusses what is known about iron translocation, especially about iron transport in the xylem, and about iron status signaling between roots and shoots. It also reviews what is known about the *FRD3* gene of *Arabidopsis thaliana*, which appears to be involved in iron translocation. The *frd3* mutant phenotype includes chlorosis and constitutive expression of iron uptake responses. These appear to be the result of mislocalization of iron in both the root and the shoot of *frd3* mutant plants. This and other evidence indicates that the FRD3 protein is likely to play a role in delivering iron to the shoot in a usable form. Specifically, FRD3 is likely to function in root xylem loading of an iron chelator or other factor necessary for efficient iron uptake out of the xylem or apoplastic space and into leaf cells. Potentially, much can be learned about both iron movement within plants and about iron status signaling from the phenotypes of *Arabidopsis* plants carrying mutations in the *FRD3* gene and from the biochemical properties of the FRD3 protein.

Key words: *Arabidopsis*; *frd3*; iron homeostasis; iron transport; xylem

1. INTRODUCTION

While much is known about how plants acquire iron from the soil, less is known about how iron moves through the plant after entering the root system. Also, since iron is both a necessary nutrient and toxic in excess, plants and all other iron-requiring organisms must carefully regulate iron uptake. Plants acquire iron through the roots but require iron throughout the plant; therefore, information about iron status must be communicated between various parts of the plant. The mechanisms used in this communication

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and those that control the root iron uptake responses are likewise largely unknown.

Genetically tractable model systems allow rapid progress in the identification of unknown components of pathways. Both forward genetic approaches that begin with a mutant phenotype and then ask what is the associated genotype, and reverse genetics that starts with a gene and asks what is the phenotype caused by mutations in that gene, are very useful in uncovering unknown mechanisms. For plant species, a widely used genetic model is the thale cress *Arabidopsis thaliana*. With its short generation time, small genome and publicly available genome sequence from two different ecotypes (*Arabidopsis* Genome Initiative, 2000; Jander *et al.*, 2002), one can isolate almost any *Arabidopsis* mutation that can be mapped in a matter of months. Additionally, null mutations in the majority of *Arabidopsis* genes are publicly available through community T-DNA mutation and sequencing projects (for example, see the TAIR website: www.arabidopsis.org). All these resources make *Arabidopsis* an ideal system for both forward and reverse genetics.

1.1 Iron translocation

In plants, once iron is taken up by the root it must be transported throughout the plant. Iron enters root epidermal cells through either the Strategy II uptake of ferric-phytosiderophore complexes used by the grasses or the Strategy I acidification, reduction, and ferrous transport pathway present in all other plants. Next, iron must be moved through the root to the central vascular cylinder where it can be loaded into the xylem and translocated to the aerial parts of the plant. All solutes must enter the vascular cylinder through a symplastic pathway because of the presence of the Casparian band, a layer of suberin-coated endodermal cells. The waxy suberin together with the endodermal cells themselves form a water and solute impermeable barrier. This separates the soil solution and the apoplast of the outer root from the apoplast of the inner root and the vascular cylinder. The vascular cylinder contains both the xylem and the phloem. The phloem is responsible for transporting metabolites from the shoot to the root, while the xylem transports water and solutes from the root to the shoot.

Due to its low solubility at neutral pH and potential to catalyze the formation of damaging oxygen radicals in unchelated forms, iron presumably moves as Fe(III) chelates within the xylem and phloem. Nicotianamine (NA) has been shown to chelate a variety of metals, including iron, copper, zinc, and manganese (Stephan *et al.*, 1996). NA complexes both Fe(II) and Fe(III), although the Fe(III)NA complex is kinetically less stable than the Fe(II)NA complex (von Wirén *et al.*, 1999). At the relatively

higher pH of the phloem, both Fe(III) and Fe(II) are predicted to be complexed with NA (von Wirén *et al.*, 1999). There is a large body of mostly correlative evidence that citrate is the major iron chelator in the xylem (reviewed in Abadía *et al.*, 2002). Work performed in the 1960's by Brown and coworkers indicated associations between iron and citrate in the xylem (reviewed in Abadía *et al.*, 2002).

It is not clear if transport within the plant differs significantly between Strategy I and II plants. The grasses synthesize a class of NA derivates, the mugineic acids, that are both excreted phytosiderophores and present in xylem exudates. One of these, 2'-deoxymugineic acid, has been predicted to be the predominant chelator at acidic xylem pH (von Wirén *et al.*, 1999). In barley, the xylem concentrations of citrate, mugineic acid, and 2'-deoxymugineic acid were all found to increase significantly under iron deficiency (Alam *et al.*, 2001). However, similar to Strategy I plants, NA is predicted to be the primary iron chelator at the more alkaline pH of the phloem (von Wirén *et al.*, 1999).

Once iron is translocated to the leaf tissue through the xylem and into the leaf apoplast, it must be taken up by the leaf cells; the molecular basis of this process remains largely unknown. Ferric chelate reductase activity has been detected in leaf cells (González-Vallejo, *et al.*, 2000, and references therein). This supports the idea that leaf iron uptake parallels the Strategy I root ferric reduction and ferrous transport. In *Arabidopsis*, the *FRO2* root ferric chelate reductase gene is a member of a small gene family (Connolly *et al.*, 2003); other members of this family may be needed to reduce iron in the shoot apoplast prior to uptake into leaf cells. Additionally, there are several predicted divalent cation uptake transporters with sequence similarity to IRT1, which is the main root ferrous uptake transporter (Guerinot, 2000; Vert *et al.*, 2002); one or more of these transporters may be responsible for iron uptake into the leaves. In addition, there are at least two other classes of uptake transporters that may be involved in this process: the AtNRAMP family and the YSL family (Curie *et al.*, 2001; Thomine *et al.*, 2000).

The tomato mutant *chloronerva* (*chln*), a NA auxotroph, has also been useful in elucidating both the role of NA in plant iron nutrition and the potential pathway of iron translocation in the plant. The phenotype of the *chln* mutant includes constitutive expression of the Strategy I root iron uptake responses, iron overaccumulation in the roots and leaf veins but intracellular underaccumulation in leaf cells, and death unless NA is supplied exogenously (Herbik *et al.*, 1996; Stephan and Scholz, 1993). Characterization of iron uptake in protoplasts from the *chln* mutant and from wild type revealed no differences in iron uptake, implying that NA is not directly involved in iron uptake (Pich and Scholz, 1991).

The *chloronerva* gene has been cloned by a map-based approach and shown to encode the tomato nicotianamine synthase; the wild-type chln protein was shown to have nicotianamine synthase activity based on *in vitro* enzyme activity of bacterially expressed protein (Ling *et al.*, 1999). The various phenotypes of the *chln* mutant have lead to the hypothesis that the Fe(II) nicotianamine complex is a signal molecule for iron status (Pich and Scholz, 1991; Scholz *et al.*, 1985). However, it is equally possible that the lack of nicotianamine simply prevents sufficient quantities of iron from getting to the cells and tissues where it is needed. Therefore, in the *chln* mutant, the iron deficiency signal, whatever its molecular nature, is constitutively sent from the shoots to the roots and the root iron uptake responses are expressed at all times.

1.2 Iron status signaling

Relatively little is known about the regulation of iron deficiency responses in *Arabidopsis* or other plant species. The pea mutants *brz* (bronze) and *dgl* (degenerative leaves) both exhibit constitutive expression of Strategy I responses and overaccumulation of iron (Gottschalk, 1987; Grusak and Pezeshgi, 1996; Kneen *et al.*, 1990; Welch and LaRue, 1990). Grafting studies, in which wild type shoots were grafted onto mutant roots and *vice versa*, indicate that there is a shoot-derived signal that controls the iron uptake responses in the root (Grusak and Pezeshgi, 1996). When wild-type shoots were grafted onto *brz* roots, the roots regained the wild type phenotype and only expressed iron uptake responses under conditions of iron deficiency. And when *brz* or *dgl* shoots were grafted onto wild type roots, the roots constitutively expressed iron deficiency responses. The fact that the behavior of the roots is determined by the genotype of the grafted shoots implies that a signal from the shoot controls the expression of iron deficiency responses in the root. This systemic signal could be a positive, iron uptake promoting signal that would be sent under conditions of iron deficiency. Alternatively, this signal could be a negative or repressive signal that would be sent by the shoots to the roots under conditions of iron sufficiency.

Additionally, a local signal has been implicated to control the morphological remodeling caused by iron deficiency. Under iron deficiency, plants increase their root surface area by one of two mechanisms. Tomato forms epidermal transfer cells with large invaginations in the cell membrane thereby increasing root epidermal surface area. *Arabidopsis*, on the other hand, increases the number and length of root hairs to increase surface area. Work with both plants in a split root system reveals that ferric chelate reductase activity is more highly expressed in iron-supplied root while

transfer cell and excess root hair formation are only observed in iron-deficient root (Schikora and Schmidt, 2001). This supports the hypothesis that ferric chelate reductase activity, as well as the other Strategy I iron uptake responses, are controlled by a systemic signal from the leaves while changes in root morphology are locally controlled.

2. PHENOTYPES OF THE *frd3* MUTANT

2.1 Mutant screening

Multiple alleles of the *frd3* mutant have been identified through a variety of screens. The first reported alleles, *frd3-1* and *frd3-2*, were identified in a screen for *Arabidopsis* mutants that continued to express ferric chelate reductase activity when grown under iron sufficient conditions (Yi, 1995); this screen was designed to identify mutations in iron sensing or signaling pathways. At approximately the same time, the *man1* mutant was identified in a screen for manganese overaccumulators (Delhaize, 1996); *man1* was later shown to be allelic to the *frd3* mutants and renamed *frd3-3* (Rogers and Guerinot, 2002). Since then *frd3-4* was identified in the *Arabidopsis* Biological Resource Center collection of chlorotic lines (E.E.R and M.L. Guerinot, unpublished) and *frd3-5* was found in an elemental analysis screen (Lahner *et al.*, 2003).

2.2 Iron localization in the *frd3* mutant

One of the most striking phenotypes of the *frd3* mutant is its alterations in iron homeostasis. Examination of divalent cation levels in the leaves of the *frd3* mutant reveals that metal levels are highly growth-condition dependent. If the *frd3* mutant is grown in nutrient media, either hydroponically or in agar plates, conditions where iron is plentiful and highly available, it accumulates higher levels of iron in its leaves than does wild type (Delhaize, 1996; Rogers and Guerinot, 2002). However, if *frd3* is grown on soil where iron is less available, its leaves contain approximately 10% less iron than wild type (Lahner *et al.*, 2003). Intracellular iron levels in the *frd3* mutant are approximately half of wild type levels, as shown by elemental analysis of protoplasts isolated from *frd3* mutant and wild-type leaf tissues (Green and Rogers, 2004). Additionally, *frd3* leaves contain much lower levels of ferritin, an iron storage protein that only accumulates under conditions of excess iron (Rogers and Guerinot, 2002). In plants, ferritin accumulates in the chloroplast and other plastids and can be used as an indirect marker of iron levels in that compartment. On the other hand, the

roots of the *frd3* mutant over accumulate a variety of metals including iron (Delhaize, 1996; Rogers and Guerinot, 2002). This iron has been shown to be intracellular based on the high levels of ferritin protein present in the mutant roots and is predominantly localized to the vascular cylinder as demonstrated by Perls' staining (Green and Rogers, 2004). Under all growth conditions, *frd3* over accumulates manganese, zinc and cadmium (Delhaize, 1996; Lahner *et al.*, 2003; Rogers and Guerinot, 2002); the manganese overaccumulation was also shown to be apoplastic (Green and Rogers, 2004).

2.3 Strategy I iron uptake responses

Perhaps as a consequence of the lower levels of intracellular iron present in its leaves, the *frd3* mutant constitutively expresses its Strategy I iron uptake responses. This includes the acidification response, ferric chelate reductase activity and the *FRO2* mRNA and *IRT1* mRNA and protein (Rogers and Guerinot, 2002). It is likely that this constitutive expression of *IRT1* and accumulation of IRT1 protein is the cause of the overaccumulation of zinc, manganese and cadmium in the *frd3* mutant. The IRT1 protein has been shown to transport all three of these metals in addition to iron (Eide *et al.*, 1996; Korshunova *et al.*, 1999; Rogers *et al.*, 2000). Additionally, *irt1* mutants, unlike wild type *Arabidopsis* and most other plants, do not overaccumulate zinc, manganese or cadmium under iron deficiency (Vert *et al.*, 2002).

2.4 Shoot iron supply in the *frd3* mutant

The phenotypes of the *frd3* mutant outlined above are consistent with the *frd3* mutant being unable to translocate iron from the root to the shoot in a form that is efficiently used by leaf cells. Additional support for this hypothesis comes from reciprocal grafting experiments performed between the *frd3* mutant and its corresponding wild type (Green and Rogers, 2004). Wild-type *Arabidopsis* seedlings, whether they are intact or have been self-grafted, do not display significant ferric chelate reductase activity, do not exhibit chlorosis and do accumulate ferritin protein in their leaf tissue after three days growth on iron sufficient media. In contrast, *frd3* mutant seedlings and self-grafts display high levels of ferric chelate reductase activity, are chlorotic and do not accumulate detectable levels of ferritin protein in their leaf tissue, even after three days growth on iron-sufficient media. As summarized in Table 16-1, in reciprocally grafted plants, the phenotype of the whole plant follows the genotype of the root, not the genotype of the shoot. For example, when a wild-type shoot is grafted onto a

frd3 mutant root, the wild-type shoot becomes chlorotic and no longer accumulates ferritin protein, an indirect measure of intracellular iron status. This is consistent with the hypothesis that the *frd3* mutant root is incapable of supplying sufficient usable iron to the wild-type shoot. Therefore, the wild-type shoot becomes iron deficient and sends an iron status signal to the roots, which constitutively express their iron uptake responses (as is shown in Table 16-1). Conversely, when a *frd3* mutant shoot is grafted onto a wild-type root, that mutant shoot is not chlorotic and does accumulate ferritin protein. This indicates that when iron is properly supplied by a wild-type root, the *frd3* mutant shoot is able to import and appropriately use that iron, as indicated by the presence of ferritin protein and the absence of chlorosis. Since the *frd3* shoot is now iron replete, it sends an iron status signal to the roots and the wild type roots repress their iron uptake responses. These grafting results indicate that FRD3 function is only needed in the root to confer a wild type phenotype; this is consistent with gene expression dating indicating that *FRD3* is only expressed in the root (Rogers and Guerinot, 2002).

Table 16-1. Reciprocal grafts were performed between wild type (wt) and *frd3*. *frd3* roots are impaired in their ability to supply iron to the shoot. Seedlings were allowed to recover for 10 days, placed on iron-sufficient medium for 3 days to repress iron uptake responses, and then assayed for ferric chelate reductase (reductase) activity, chlorophyll levels (chlorosis) and ferritin protein levels (ferritin).

Shoot	Root	Reductase	Chlorosis	Ferritin
wt (ungrafted)		no	no	yes
wt	wt	no	no	yes
frd3	wt	no	no	yes
wt	*frd3*	yes	yes	no
frd3	*frd3*	yes	yes	no
frd3 (ungrafted)		yes	yes	no

3. THE *FRD3* GENE AND PREDICTED PROTEIN PRODUCT

The *FRD3* gene was cloned by a map-based approach and shown to encode a protein belonging to the MATE (multidrug and toxin efflux) family of transmembrane exporters (Rogers and Guerinot, 2002). The mapping data was confirmed by complementation of the mutant phenotypes by genomic DNA containing only the predicted *FRD3* gene and by the presence of sequence changes in all mutant alleles (Rogers and Guerinot, 2002). *FRD3* is predicted to encode an integral membrane protein 526 amino acids long. Topology prediction programs and comparisons with other MATE family members indicate that the FRD3 protein likely contains 12 transmembrane

domains and is oriented with the N- and C-termini on the cytoplasmic side of the membrane. The cytoplasmic location of the C-terminus has been confirmed experimentally (L.S. Green and E.E.R, unpublished).

Comparison of the *FRD3* genomic sequence and a full length *FRD3* cDNA sequence (Genbank accession number AF448231) reveals that the *FRD3* gene has 13 exons and 12 introns. Most unusually, the first intron is in the 5' untranslated region and is almost 2.6 kb in length; this is much larger than the approximately 170 bp average for *Arabidopsis* introns (*Arabidopsis* Genome Initiative, 2000). Long introns in other *Arabidopsis* genes have been shown to play important roles in the regulation of gene expression (Jeon *et al.*, 2000). The regulatory role of the first intron in *FRD3* has yet to be shown. However, it is interesting to note that expression of the *FRD3* cDNA behind a strong constitutive promoter in *frd3-1* only complements the mutant phenotype in approximately 10% of the lines (E.E.R., unpublished).

The sequence changes detected in all the *frd3* mutant alleles are summarized in Table 16-2. The mutant phenotypes of *frd3-1, 3-2, 3-3* and *3-5* are indistinguishable.

Table 16-2. Sequence changes in *frd3* mutant alleles. The allele number, mutagen used, DNA, RNA and predicted protein sequences changes are shown.

Allele	Mutagen	DNA/RNA	Protein	Reference
3-1	EMS	C to A	Asp to Ala	Rogers and Guerinot, 2002
3-2	EMS	1 bp deletion	premature stop codon	Rogers and Guerinot, 2002
3-3	EMS	G to A in splice site; intron retained	premature stop codon	Rogers and Guerinot, 2002
3-4	EMS	C to T in intron; intron retained in most mRNAs	premature stop codon	E.E.R., unpublished
3-5	fast neutron	7 bp deletion	premature stop codon	Lahner, *et al*., 2003

However, *frd3-4* has a less severe and rather variable phenotype (E.E.R., unpublished). The C to T base pair change is in the middle of the predicted branch site of the third intron (Lim and Burge, 2001). In *frd3-4*, the third intron is retained in the vast majority of individual *frd3* mRNAs. Sequencing of RT-PCR products from *frd3-4* does detect a small number of properly spliced mRNAs that are predicted to encode a wild-type FRD3 protein (E.E.R., unpublished). Presumably, this low level of wild type mRNA and protein causes the less severe mutant phenotype in *frd3-4.*

The C to A transversion in *frd3-1* causes substitution of aspartic acid for the alanine residue at position 54 in the first transmembrane domain (Rogers and Guerinot, 2002). While it is a little unusual for such a missense mutation to have as severe a phenotype as the premature protein truncations found in

frd3-2 and *frd3-3*, which both eliminate at least half of the predicted protein product, it is possible that a negatively charged amino acid in the first transmembrane domain prevents proper insertion of the protein into the membrane and leads to its degradation.

3.1 *FRD3* gene expression and protein localization

Expression of the *FRD3* gene has been detected in root tissue but not in leaves (Rogers and Guerinot, 2002); this is consistent with the FRD3 protein playing a role in xylem loading. Unpublished but publicly available *Arabidopsis* microarray results have detected high levels of *FRD3* expression in xylem tissues and lower levels in stamens (http://affymetrix.arabidopsis.info/narrays/); no phloem-specific arrays are available. In root tissue, iron deficiency induces the *FRD3* mRNA level to approximately twice its iron-sufficient expression (Rogers and Guerinot, 2002).

Examination of FRD3-GFP and FRD3-FLAG fusion proteins indicates that the protein is expressed in the root vascular cylinder, specifically in the pericycle and cells surrounding the xylem and phloem (Green and Rogers, 2004). Unfortunately, these cells are too small to clearly distinguish membrane localization of the FRD3 protein. The FRD3 protein is predicted to localize to the plasma membrane (Rogers and Guerinot, 2002); the immunofluorescence data available is consistent with a plasma membrane localization for at least some of the FRD3 protein (Green and Rogers, 2004).

4. OTHER MEMBERS OF THE MATE FAMILY

Functionally characterized members of the MATE family appear to efflux low molecular weight organic compounds from the cell cytoplasm either out of the cell or into a subcellular compartment (for a summary, see Rogers and Guerinot, 2002). There are at least 56 MATE family members in *Arabidopsis* (Li *et al.*, 2002; Rogers and Guerinot, 2002). They play roles in processes as diverse as salicylic acid localization, flavonoid transport, and detoxification. Therefore it is very difficult to predict a function for a newly discovered MATE protein simply based on protein sequence similarity to a previously characterized MATE family member.

The *Arabidopsis* gene most similar to *FRD3* is At1g51340 which is 57.8% identical at the amino acid level to FRD3. It has been named *FRDL* (for *FRD3*-like) (Rogers and Guerinot, 2002). A T-DNA insertion just upstream of the ATG completely eliminates *FRDL* mRNA; however, *Arabidopsis* plants homozygous for this T-DNA insertion have no changes

in iron or other metal homeostasis nor alterations in the regulation of their iron uptake responses (E.E.R., unpublished). This indicates that in spite of *FRDL*'s similarity to *FRD3*, the *FRDL* gene product does not play a necessary and non-redundant role in iron translocation, homeostasis or the regulation of iron uptake responses.

A number of ESTs (expressed sequence tags) with significant similarity to *FRD3* have been identified in soybean, rice and *Medicago truncatula*. The *M. truncatula* TC 30920 has been identified as a nodule-specific gene with 76.7% amino acid sequence identity to FRD3 over a stretch of approximately 60 amino acids (Fedorova *et al.*, 2002). No specific roles in iron translocation or homeostasis have been proposed for this *M. truncatula* gene. Additionally, three rice genes, *OsFRDL1, 2,* and *3*, have been identified (Inoue *et al.*, 2004). These predicted proteins share 51%, 50%, and 41% amino acid identity, respectively, with FRD3 but 53%, 56%, and 43% identity to FRDL. *OsFRDL3* is also 52% identical to At4g38380, an uncharacterized MATE gene from *Arabidopsis*. Further characterization of these rice genes is needed to establish a role for them in iron translocation or homeostasis.

5. MODEL OF FRD3 ACTION

Putting together all the data about the phenotypes of the *frd3* mutant plants and the expression pattern, probable membrane localization, and predicted biochemical function of the FRD3 protein, leads us to this question: how can a putative effluxer of low molecular weight compounds that is expressed around the root vasculature affect iron localization in the shoot?

Iron reaches the shoot tissue in sufficient amounts in the *frd3* mutant. Shoots of the *frd3* mutant grown under conditions of low iron availability on soil, have iron levels that are only about 10% lower than in wild type (Lahner *et al.*, 2003). This evidence that significant amounts of iron do reach the shoot is consistent with iron being loaded into the xylem appropriately in the *frd3* mutant. Therefore, we do not believe that the FRD3 protein is an iron transporter. Because the *frd3* mutant has very high levels of iron in the vascular cylinder of the root, the mutant appears to have problems in moving iron through the xylem and in getting iron out of the xylem, into the shoot symplast, and then to the chloroplasts. However, it is doubtful that FRD3 acts in the shoots since *FRD3* is not expressed to detectable levels in the leaves. The reciprocal grafting experiments also indicate that functional FRD3 protein is not needed in the shoots for wild-type iron localization and signaling.

Given *FRD3*'s expression in cells surrounding the root vasculature, we hypothesize that FRD3 effluxes into the xylem a low molecular-weight compound that is necessary for correct iron unloading from the xylem in the shoot. This compound most probably is an iron chelator. The chelator widely thought to bind iron in the xylem is citrate. The FRD3 protein does contain motifs essential for the function of a number of bacterial citrate transporters (Timothy P. Durrett and E.E.R., unpublished). Experiments to examine xylem citrate levels in the *frd3* mutant and to test FRD3's ability to transport citrate are ongoing.

Nicotianamine (NA) is another molecule that has been implicated in iron homeostasis and long distance iron transport in plants. It appears that NAS (nicotianamine synthase) genes are expressed and NA is synthesized throughout the plant (Ling *et al.*, 1999; Suzuki *et al.*, 1999). The transporter responsible for loading NA into the vasculature has not been identified; this is a possible function for FRD3. The *frd3* mutant has wild-type copper levels (Lahner *et al.*, 2003). However, NA-less *chln* mutant has alterations in copper homeostasis as well as in iron homeostasis (Herbik *et al.*, 1996; Pich *et al.*, 1994). This difference makes it unlikely that the FRD3 protein is involved in NA transport. Whatever the substrate of FRD3 may be, it is likely to have the following characteristics: it will be synthesized and loaded into the xylem in the root and it will be necessary for iron mobility in the xylem or iron unloading from the xylem and/or shoot apoplast.

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Chapter 17

FERRITINS AND IRON ACCUMULATION IN PLANT TISSUES

JEAN-FRANÇOIS BRIAT, FRANÇOISE CELLIER and FREDERIC GAYMARD

Biochimie et Physiologie Moléculaire des Plantes. Centre National de la Recherche Scientifique (UMR 5004), Institut National de la Recherche Agronomique, Université Montpellier 2, École Nationale Supérieure d'Agronomie, 2 Place Viala, F-34060 Montpellier cedex 2, France. E-mail: briat@ensam.inra.fr

Abstract: At physiological pH, iron tends to precipitate as insoluble forms. Furthermore, its redox cycling participates in activation of reduced forms of oxygen through Fenton chemistry, leading to cellular damage and cell death. As a consequence of these properties, plants have evolved mechanisms to control iron uptake and storage. A class of multimeric proteins, the ferritins, acts as an iron buffer inside the cell by storing this ion in a soluble, non-toxic and bioavailable form. These proteins are present in plants, animals, fungi and bacteria. In plants, they are localized within plastids, and their synthesis is regulated by developmental and environmental factors, such as iron excess or oxidative stress. In particular, iron overload transcriptionally activates some ferritin genes. Both an abscissic acid-dependent and an abscissic acid-independent pathway are involved in this iron response. The latter involves some phosphorylation and dephosphorylation events, and specific *cis*-regulatory elements have been evidenced in some ferritin promoter sequences. Ferritin expression has been experimentally deregulated in transgenic plants by overexpressing them under the control of various heterologous promoters. As a consequence of ferritin accumulation in these transgenic plants, an increase in leaf or seed iron concentration by 2- to 3-fold was reported, concomitantly with an increase in root ferric reductase and root H^+ - ATPase activities, two key determinants of iron uptake by dicotyledonous plants. This leads to propose a biotechnological approach to treat human iron deficiency estimated to affect 30% of the world population. However, the iron–enriched phenotype of plants over-accumulating ferritins seems to be highly soil-type dependent.

Key words: biofortification; iron homeostasis; oxidative stress

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1. INTRODUCTION

Iron mining from the soil by plant roots enables its entry into the biosphere where it can play its numerous essential functions. In plants, they include carbon, sulfur and nitrogen assimilation that takes place in specific plant sub-cellular organelles, the plastids, and require numerous proteins coordinating Fe as heme or Fe-S clusters. Accumulation of Fe in the various plant tissues during growth and development, throughout the life cycle, is a dynamic process resulting from an integrated regulation of the expression of the various genes encoding proteins acting in the transport and storage of Fe. These processes depend upon the plant species and genotypes considered, and are deeply influenced by environmental cues.

Due to their immobility, plants are highly dependent on their environment and then need a tight regulation of Fe uptake, transport in various organs, and storage to ensure an optimal development by preventing both Fe deficiency and toxicity. Various transporters are required to achieve these Fe fluxes (Curie and Briat, 2003). Iron storage takes place in the apoplasmic space, between the plasma membrane and the cell wall of plant cells, and perhaps in the vacuoles, where low pH and high organic acid concentrations represent optimal conditions for Fe deposit (Briat and Lobréaux, 1998). Ferritins are Fe storage proteins located in the plastids of plant cells. They are also part of these mechanisms by their capacity to store up to 4,500 Fe atoms in their cavity in a soluble and bioavailable form (Harrison and Arosio, 1996). This review will summarize our present knowledge of this class of proteins, with a particular emphasis on (i) the various regulations modulating its expression, and (ii) the potential biotechnological applications from manipulating its expression.

2. FERRITINS AND IRON HOMEOSTASIS IN VEGETATIVE ORGANS DURING PLANT LIFE CYCLE

2.1 Leaves

The leaf is a major sink for Fe accumulation in plants, and chloroplasts contain 80% of the metal present in this organ. It is consistent with the major function of this organ, photosynthesis, a reaction requiring Fe in the many Fe-containing proteins of the electron transfer chain of the thylakoid membranes. The Fe storage protein ferritin accumulates mainly in non-green plastids, like etioplasts or amyloplasts, while low level of this protein is found in mature chloroplasts where the photosynthetic process is active. The

distribution of Fe in leaves depends on the developmental stage. The developmental control of ferritin synthesis in leaves is evidenced by an increase of ferritin abundance in developing and in senescent leaves (Briat and Lobréaux, 1997). These results have been obtained by immunodetection of ferritin subunits in protein extracts during the life cycle of pea plants (Lobréaux and Briat, 1991). Ferritins were only detected in leaves of young plantlets, and remained undetectable in mature leaves of adult plants at later developmental stages. These data have been further documented by using maize as a model to study ferritin synthesis during leaf development (Theil and Hase, 1993). In this plant, the leaf grows from the basal part, creating a cell age gradient from the base to the tip of the leaf. By an immunodetection approach, high ferritin levels were detected in the young basal section of the leaf and in the tip, the older part of the leaf. On the opposite, a very low level of ferritin was present in the central section of this organ. This leaf zone has the higher chlorophyll content, and the detection of the phosphoenolpyruvate carboxylase enzyme in this region, a marker of photosynthetic activity in C4 plants like maize, proves that the photosynthetic process is active in these cells. These results demonstrate the developmental regulation of ferritin synthesis in leaves. Such data are in complete agreement with previous electron microscopy observations, showing that ferritins were synthesized in meristematic zones and apices, in leaf primordia, and disappeared during bud development (Marinos, 1967; Seckback, 1982). All these observations suggest that ferritin would be a Fe source at early stages of development for the synthesis of Fe containing proteins involved in photosynthesis. This hypothesis is consistent with the fact that ferritins are present in young leaves or etiolated leaves containing non photosynthetic etioplasts, and then become undetectable in photosynthetic or de-etiolated leaves (Lobréaux and Briat, 1991; Seckback, 1982). The high ferritin concentration in maize leaf tips could be linked to older cell senescence.

Ultrastructural studies have clearly established that ferritin accumulates in senescing tissues (Seckback, 1982). Furthermore, a *Brassica napus* cDNA corresponding to a mRNA encoding a ferritin subunit has been cloned as a senescence induced mRNA (Buchanan-Wollaston and Ainsworth, 1997). The neo-synthesis of plant ferritins at later stages of development, during leaf senescence has also been reported. The regulatory mechanisms controlling ferritin synthesis during leaf development are still unknown, but it has been established in soybean and maize that there is no direct correlation between the levels of ferritin subunits and mRNA (Ragland *et al.*, 1990). Ferritin mRNA was detected in mature leaves where ferritin subunits were not detected by western blot experiments, demonstrating that post-trancriptional controls are involved in the regulation of ferritin synthesis in this organ. More recently, however, it has been reported that

cis-regulatory element(s) are present within 1.4 kbp promoter sequence of the *AtFer1* ferritin gene from *Arabidopsis*, that are necessary to activate its expression during age-dependent senescence. These regulatory elements are different of the IDRS box (see below) known to regulate the Fe-dependent expression of the *AtFer1* gene (Tarantino *et al.*, 2003). In contrast, dark-induced senescence promotes *AtFer1* gene activation through the IDRS box. In that latter case, Fe release during the disorganization of the photosynthetic apparatus, and the requirement to store this metal, could trigger the transcriptional expression of *AtFer1*.

2.2 Roots and nodules

Iron concentration in roots is much lower than in leaves. Nevertheless ferritin is also present in the non-green plastids of this organ. In *Arabidopsis*, the *AtFer1* gene has been reported to be expressed close to the root tip in the endoderm cell layer, and at the emergence of secondary roots (Tarantino *et al.*, 2003). It is therefore plausible that ferritin may act as Fe buffer in non-green plastids in the endodermal cells after Fe uptake at the epidermis and cortex levels. In roots, ferritins would therefore be involved in modulating the flux of Fe to the upper part of the plant, through loading of the xylem sap by still uncharacterized transporters.

Plants from the legume family specifically develop a symbiosis with some soil bacteria enabling nitrogen fixation. This symbiotic process takes place in specific root structures, the nodules, within the cortical cells. In nodules, bacteria evolved into bacteroids able to reduce atmospheric nitrogen into ammonia. This process requires some essential Fe containing proteins such as nitrogenase and leghemoglobin (Tang *et al.*, 1990). Iron uptake by the bacteroids within the nodule occurs through three mechanisms: (i) Fe(III) chelated to organic acids like citrate is transported across the peribacteroidal membrane prior to accumulation within the peribacteroidal space (Levier *et al.*, 1996; Moreau *et al.*, 1995) where it will bind to bacterial-type siderophores (Wittenberg *et al.*, 1996), (ii) Fe(III)-chelate reductases are active at the peribacteroidal membrane and uptake of Fe(III) in isolated symbiosomes is stimulated by NADH (Levier *et al.*, 1996), and (iii) Fe(II) is also transported across the peribacteroidal membrane (Moreau *et al.*, 1998) likely through the GmDMT1 transporter (Kaiser *et al.*, 2003) which belongs to the NRAMP family.

Bacteroid nitrogenase is directly involved in nitrogen assimilation, and requires low oxygen concentration. The protection against oxygen is achieved by the leghemoglobin, another Fe protein synthesized by the plant. In the interaction between soybean and a *Bradyrhizobium* strain, plant ferritin has been shown to accumulate at early stages of nodule development

resulting from this interaction (Ko *et al.*, 1987). Ferritin levels decrease with the appearance of nitrogenase and leghemoglobin when the nodule becomes mature for nitrogen fixation (Bergersen, 1963). When soybean plants are cultured in the presence of a mutant strain of *Bradyrhizobium* unable to develop functional nodules, ferritin structures are detected at any stages of development of these nodules (Ko *et al.*, 1987). In senescing nodules of *Lupinus luteus*, ferritin is re-synthesized through the expression of 2 out of the 3 lupine ferritin genes (Strozycki *et al.*, 2003). These data suggest again that Fe would be transiently stored in ferritins and used for the accumulation of Fe containing proteins. Ferritin mRNA levels have also been investigated during nodulation. This study reveals that, as for leaf development, no strict correlation between ferritin subunit and mRNA levels is observed (Ragland and Theil, 1993) suggesting that some posttranscriptional events would occur in the regulation of ferritin level during nodule development.

3. FERRITINS AND IRON ACCUMULATION IN SEEDS

Studies on the fate of Fe during the course of vegetative organs growth and development have evidenced the dynamic nature of this process. Iron concentration changes within organs, in a tissue specific manner, during the course of development. The role of ferritin as a transient Fe buffer for important Fe-dependent processes like photosynthesis and nitrogen fixation has been well documented in these developmental processes. Ferritins are also key proteins in long-term Fe storage, as evidenced by seed formation studies. An important amount of Fe is stored in pea seeds, and an increase in Fe uptake by the roots occurs at early stages of seed development (Lobréaux and Briat, 1991). Iron is also remobilized from vegetative organs to the seed. For example, it has been documented that leaf Fe can account for 20-30% of the total seed Fe content (Grusak, 1994; Hocking and Pate, 1978). In legumes such as soybean, it has been suggested that 40 to 60% of the seed Fe could come from nodules (Burton *et al.*, 1998). Root nodules of legumes have higher concentrations of Fe than other vegetative organs. Therefore an active remobilization of nodule Fe to the seed could explain why legume seeds have high concentration of Fe relative to other plants, making legume seeds a rich source of dietary Fe.

Seed Fe is used during germination for the plantlet development. Immunodetection experiments revealed that ferritin subunits accumulated in seed during their maturation and remained present in dry seeds (Lobréaux and Briat, 1991). This accumulation occurred in the embryo, and no ferritins were detected in the seed coat (Lobréaux and Briat, 1991; Marentes and

Grusak, 1998). The amount of Fe stored inside ferritins was estimated to be 92% of the total seed Fe content (Marentes and Grusak, 1998), suggesting that this protein is the major form of Fe storage in seeds. Whether this Fe pool is used only for plastid development or is also transferred to the cytosol remains to be determined. During germination, ferritins are degraded and Fe is used for the growth of the seedling. This process would involve a mechanism that controls the stability of the ferritin protein leading to its degradation when Fe is released from the mineral core. A model has been proposed based on the observation that the immunodetection pattern of pea seedling ferritin is similar to the pattern of ferritin degraded by free radical during *in vitro* Fe exchanges (Lobréaux and Briat, 1991). When Fe release is induced from purified pea seed ferritins by incubation in the presence of ascorbate or light, a progressive degradation of the protein is detected (Laulhère *et al.*, 1989; Laulhère *et al.*, 1990). This degradation is initiated by Fe-dependent free radical cleavages at the aminoterminus of the ferritin subunit (Laulhère *et al.*, 1989; Laulhère *et al.*, 1990; Lobréaux *et al.*, 1992a). It has been hypothesized that such a mechanism occurs during seedling germination. When Fe is released from the ferritin, free radical cleavages could alter the protein shell, which would be then degraded by proteases.

4. REGULATION OF FERRITIN GENE EXPRESSION

Plant and animal ferritins have evolved from a common ancestor gene as suggested by amino acid sequence comparison of ferritin subunits (Andrews *et al.*, 1992). However, in the two kingdoms, ferritins are localized in different compartments; plant ferritins are localized in the plastids (Lescure *et al.*, 1991; Van der Mark *et al.*, 1983), while animal ferritins are cytosolic proteins (Harrison and Arosio, 1996). This specific compartimentalization, and the requirement to adapt the ferritin content to plant development, as described above, suggest that some plant specific pathways would be involved in the regulation of the ferritin subunit and mRNA levels in this organism. It is now clear that even the Fe dependent regulation of animal ferritin genes is not conserved in plants since it has been demonstrated that Fe resupply to Fe starved soybean cell suspension cultures induced ferritin mRNA accumulation controlled at the transcriptional level (Lescure *et al.*, 1991). Although important data documenting the regulation of ferritin synthesis have been obtained with maize (Lobréaux *et al.*, 1992b; Lobréaux *et al.*, 1993; Petit *et al.*, 2001a; Savino *et al.*, 1997) and soybean (Lescure *et al.*, 1991; Wei and Theil, 2000), *Arabidopsis* is now offering the best opportunity to exhaustively characterize the differential expression of the

four members of the ferritin gene family present in the genome of this model species (Petit *et al.*, 2001b).

4.1 Abscissic acid-dependent regulation of ferritin gene expression in response to iron

Iron starvation of plants leads to chlorosis symptoms resulting from chlorophyll deficiency and impaired photosynthesis (Briat *et al.*, 1995). Under such conditions, root Fe uptake systems are induced in order to enhance Fe acquisition and to restore the physiological integrity of chloroplasts (Briat and Lobréaux, 1997). Indeed, addition of an excess of Fe in the culture medium of Fe starved plantlets promoted a large Fe influx throughout the plant (Lobréaux *et al.*, 1992b), leading to Fe translocation into the leaves within 3 hours to restore the essential photosynthetic process in chloroplasts (Branton and Jacobson, 1962; Young and Terry, 1982). During this period of regreening which takes about 24 to 48 hours, ferritins are used as a safe Fe buffer and transiently store the Fe required for the synthesis of Fe containing proteins. These experimental conditions have been used as a model to better understand Fe-regulated ferritin synthesis in plants. Ferritin mRNA are already detectable in maize leaf plantlets 3 hours after Fe resupply. The level of transcript accumulated reaches a maximum 6 and 24 hours after the treatment in leaves and roots, respectively, and then gradually decreased (Lobréaux *et al.*, 1993). The increase in ferritin mRNA abundance precedes the accumulation of ferritin subunits, for which the maximum level was detected 24 hours after the addition of Fe. Then, a gradual decrease of ferritin content is observed, consistent with a transient Fe buffer function of this protein during the regreening of plants (Lobréaux *et al.*, 1992b). In maize, part of this response occurring during recovery from Fe deficiency has been shown to be mediated by an abscissic acid (ABA)-dependent pathway (Lobréaux *et al.*, 1993). Among the two subclasses of ferritin genes identified in maize so far, only *ZmFer2* was shown to be responsive to ABA, consistent with the observation that this gene is also up-regulated in response to drought stress (Fobis-Loisy *et al.*, 1995), and that the maize ferritin protein accumulates in response to water deficit (Riccardi *et al.*, 1998). In *Arabidopsis*, the *AtFer2* gene is likely to be the orthologue of the maize *ZmFer2* ferritin gene, since this latter is the only one among the four *Arabidopsis* ferritin genes to be induced in response to exogenous ABA application, and to be specifically expressed in seeds (Petit *et al.*, 2001b).

4.2 Iron-regulated expression of ferritin genes independently of the abscissic acid pathway

In maize, during recovery from Fe-deficiency, it was observed that part of the response leading to ferritin synthesis occurs through an ABA-independent pathway. Indeed it was shown that another gene, called *ZmFer1*, was activated in response to an Fe excess treatment through an ABA-independent pathway (Fobis-Loisy *et al.*, 1995). The *ZmFer1* promoter sequence was fused to ß-glucuronidase or luciferase reporter genes and these chimeric constructs were expressed transiently in maize cell suspension cultures under various conditions. It was shown that the Fe activation of *ZmFer1* expression was antagonized by antioxidants such as glutathione or N-acetyl cysteine, as well as by okadaic acid and caliculin A, two inhibitors of PP2A-type phosphatases (Savino *et al.*, 1997). It is therefore hypothesized that the pathway transducing the "Fe excess" signal leading to the expression of the *ZmFer1* gene involves oxidative stress and phosphorylation/dephosphorylation events. The maize cell system was also used to analyse the *cis*-regulatory elements found within 1.4 kbp of the *ZmFer1* promoter. Serial deletions and site directed mutagenesis enabled to characterize a 15 bp sequence named IDRS (Iron Dependent Regulatory Sequence) within the proximal part of the promoter of the *ZmFer1* ferritin gene, necessary for the Fe-dependent expression of this gene (Petit *et al.*, 2001a). A mutated IDRS within the *ZmFer1* promoter led to the up-regulation of a reporter gene in absence of Fe, at a level comparable to what is observed with the wild-type IDRS in response to excess Fe. This result indicates that the IDRS is involved in the repression of *ZmFer1* gene expression under Fe-deficient conditions. In *Arabidopsis*, the *AtFer1* gene was shown to be the ortholog of the maize *ZmFer1* gene (Gaymard *et al.*, 1996). *AtFer1* gene expression in response to Fe excess is ABA-independent, and antagonised by antioxidants. The *AtFer1* promoter region contains a functional IDRS, as shown in a transient assay using *Arabidopsis* cell suspension cultures. Furthermore, point mutations within the IDRS in a 1.4 kbp *AtFer1* promoter region de-repress reporter gene expression in transformed transgenic plants under Fe-deficient conditions (Petit *et al.*, 2001a). The IDRS sequence has also been shown to be required for dark-induced senescence activation of the *AtFer1* expression but not for age-dependent senescence induction (Tarantino *et al.*, 2003). Using a GUS reporter gene strategy, the tissue localisation of *AtFer1* expression was determined. GUS staining of plants grown under standard Fe conditions revealed that *AtFer1* promoter activity in leaves is observed mainly in the vicinity of the vessels and mutations within the IDRS do not alter this localization. In roots, the *AtFer1* promoter activity was restricted to the

endoderm. However, IDRS mutagenesis resulted in an extension of the promoter activity to the root cortex and epidermis (Tarantino *et al.*, 2003). These results indicate that, under standard Fe nutrition conditions, the IDRS could be involved in the repression of expression of the *AtFer1* gene in the cortex and epidermal cells. In the absence of a functional IDRS, *AtFer1* gene repression would not occur anymore, leading to extend the root expression territories of this gene. Whether the IDRS function in cell/tissue specificity of expression is Fe-dependent or not remains to be determined.

Kinetics of *Arabidopsis AtFer3* transcript accumulation in response to Fe overload are very similar to the one of the *AtFer1* gene, both in roots and shoots. In contrast, the *AtFer2* gene is not induced by Fe excess and *AtFer4* mRNA abundance increased only in leaves with a different kinetics than *AtFer1* and *3* transcripts (Petit *et al.*, 2001b). The comparison of the promoter sequence of the four *Arabidopsis* ferritin genes revealed the presence of an IDRS-like element in the same promoter region of *AtFer2*, *3* and *4* ferritin genes than the functional IDRS characterized in the *AtFer1* promoter. However, it remains to be determined whether or not these IDRS-like participate to the regulation of expression of *AtFer2*, *3* and *4* genes.

Beside the IDRS, another *cis*-regulatory element than the IDRS, named FRE (Fe Responsive Element), has also been characterized in the promoter region of a soybean ferritin gene (Wei and Theil, 2000); no such element can be observed in any of the four *Arabidopsis* ferritin genes (Petit *et al.*, 2001b). So far, no *trans*-acting factors interacting with these *cis*-regulatory sequences and involved in plant ferritin gene expression have been characterized.

4.3 Oxidative stress and ferritin gene expression

Iron reactivity with oxygen produce free radicals (Briat, 2002). Therefore Fe storage and buffering is a key element of the defense mechanisms evolved by living organisms to cope with oxidative stress. Ferritins are playing an active role in these mechanisms because of their ability to store thousands of Fe atoms in a safe form (Harrison and Arosio, 1996). Indeed ferritin over-expression in transgenic plants has been shown to protect against paraquat promoted oxidative stress (Deak *et al.*, 1999; Van Wuytswinkel *et al.*, 1999; Zer *et al.*, 1994). Furthermore, pro-oxidant treatments such as H_2O_2, NO or ozone applications as well as high light intensity are known to induce ferritin synthesis (Gaymard *et al.*, 1996; Lobréaux *et al.*, 1995; Murgia *et al.*, 2001; Murgia *et al.*, 2002; Tarantino *et al.*, 2003). However, whether the regulation of ferritin expression in response to Fe excess and oxidative stress occurs through common, independent or branched pathways is still unclear.

Maize *ZmFer1* and *Arabidopsis AtFer1* ferritin genes have been shown to be induced both by Fe excess and H_2O_2 treatments (Petit *et al.*, 2001b; Savino *et al.*, 1997). The effect of both of these inducers has been shown to be antagonised by Ser-Thr phosphatase inhibitors (Savino *et al.*, 1997, Arnaud, Cellier, Briat and Gaymard, unpublished results). The increase of *AtFer1* transcript abundance in response to Fe and H_2O_2 treatments are additive, suggesting that Fe- and H_2O_2-mediated regulation of *AtFer1* expression are mediated through different pathways (Arnaud, Cellier, Briat and Gaymard, unpublished results). Consistent with this hypothesis, it has been reported that the IDRS was not involved in the regulation of *ZmFer1* expression in response to H_2O_2 (Petit *et al.*, 2001a), nor in the Fe regulation of the *Arabidopsis AtAPX1* gene encoding an H_2O_2 scavenging cytosolic ascorbate peroxidase (Fourcroy *et al.*, 2004). On the other hand, NO has been shown to be involved in the transduction pathway between the "Fe excess" signal and *AtFer1* promoter de-repression, in an IDRS-, Ser-Thr phosphatase-dependent manner (Murgia *et al.*, 2002). Further work will be needed to decipher the intricate mechanisms controlling the expression of plant genes involved in the control of Fe/oxygen interactions which potentially lead to oxidative stress.

5. FERRITIN OVEREXPRESSION IN TRANSGENIC PLANTS AND ITS CONSEQUENCES

Iron accumulation in various plant tissues is under genetic control and this is evidenced by alteration of Fe homeostasis either in plant mutants altered in Fe signalling or in transgenic plants overexpressing ferritin ectopically.

Our knowledge of the role that ferritins play in plant physiology is still very limited. Their functions have been recently addressed by an approach based on their over-expression in transgenic plants, either in the plastids (their natural cytological localization) or in the cytoplasm, to evaluate the consequences of such a deregulation on plant development and physiology (Deak *et al.*, 1999; Goto *et al.*, 1999; Van Wuytswinkel *et al.*, 1999). An illegitimate ferritin accumulation was obtained in leaves and in seeds. Although no major phenotypic alterations were reported to occur in these transgenic plants, in tobacco leaves grown *in vitro* on a media containing 25 μM Fe(III)-EDTA yellow zones were observed, consistent with a 20% decrease in chlorophyll concentration. Indeed, in these plants, some chloroplasts had an altered sub-structure with diffused thylakoids, and large stromal areas with very weak electron density (Briat *et al.*, 1999).

Iron and oxygen metabolisms can interact to promote oxidative stress. Therefore, Fe sequestration in ferritin of transformed plants could have a beneficial effect against Fe-mediated oxidative stress. Methylviologen, the active molecule of the herbicide paraquat, acts by promoting an oxidative stress in the chloroplast, leading to proteolysis, lipid peroxidation and ultimately to cell death (Dodge, 1994). The toxic effect of methyl viologen requires free Fe to take place, and can be antagonized by Fe chelators such as desferrioxamine (Korbaschi *et al.*, 1986; Zer *et al.*, 1994). Indeed plants overexpressing ferritin are more resistant to methylviologen toxicity, confirming that the transgenic ferritins were functional *in vivo* - *i.e.* able to sequester Fe atoms (Deak *et al.*, 1999; Van Wuytswinkel *et al.*, 1999). However, it has been documented in animal cells, that ferritin can act either as anti- or pro-oxidant (Cairo *et al.*, 1995). Therefore, the increased resistance to paraquat treatment mentioned above could have also resulted, at least in part, from a general activation of plant defense against oxidative stress generated in response to illegitimate accumulation of ferritin in leaves. This point was addressed by measuring various enzyme activities involved in oxygen detoxification in leaf discs of control tobacco plants, and tobacco plants overexpressing ferritin. All the enzyme activities measured (catalase, ascorbate peroxidase, guaiacol peroxidase and glutathione reductase) were indeed increased by 1.5- to 3-fold in the ferritin overexpressors (Briat *et al.*, 1999). Therefore, although resistant to methylviologen treatment, transgenic tobacco plants overexpressing ferritin experience an oxidative stress.

The major consequence of the ferritin accumulation in transgenic plants was to increase leaf or seed Fe concentration by 2- to 3-fold (Goto *et al.*, 1999; Van Wuytswinkel *et al.*, 1999), concomitantly with an increase in root ferric reductase and root H^+ - ATPase activities (Vansuyt *et al.*, 2000; Vansuyt *et al.*, 2003; Van Wuytswinkel *et al.*, 1999), two key determinants of Fe uptake by dicotyledonous plants (Curie and Briat, 2003). This can be explained by the increased Fe storage capacity of the ferritin overexpressing plants in which excessive Fe sequestration disturb the metabolism, driving leaf physiology towards an Fe deficient state. As a consequence the transgenic plants, sensing an Fe deficiency, logically activate the Fe uptake systems (Curie and Briat, 2003). Such a situation of increased Fe uptake in plants which sense their Fe status as deficient whereas they paradoxically accumulate too much Fe has already been described in the case of the *brz* and *dgl* pea mutants (Grusak and Pezeshgi, 1996; Marinos, 1967), and of the *chloronerva* tomato mutant (Ling *et al.*, 1999). However, the mechanism of Fe over-accumulation in these mutant plants differs regarding ferritin synthesis, which was observed to be activated in *brz* and *dgl* pea mutants (Becker *et al.*, 1998) but not in *chloronerva* tomato mutant (Becker *et al.*, 1995).

6. IRON BIOFORTIFICATION: A CRITICAL EVALUATION OF THE BIOTECHNOLOGICAL USE OF PLANT FERRITIN OVEREXPRESSORS

Iron deficiency is the major cause of anaemia, and it is estimated to affect 30% of the world population. Symptoms associated with this health problem can be severe, including increased susceptibility to infections and retardation of mental and psychomotor development and of growth. One of the major causes of this nutritional disorder in third world countries is malnutrition. A biotechnological approach has been recently proposed to treat human Fe deficiency. As mentioned above, overexpression of the Fe storage protein ferritin in transgenic plants has been achieved leading to a three fold increase in leaf and seed Fe content (Goto *et al.*, 1999; Van Wuytswinkel *et al.*, 1999). Similarly, oral administration of plant ferritin has been shown to be an efficient Fe source to relieve rat anaemia (Beard *et al.*, 1996). These data led to the proposal that feeding humans safely with enough Fe directly within their diet could become possible by using Fe fortified transgenic plants overexpressing ferritin (Goto *et al.*, 1999). However, it is well known that Fe uptake by plant roots results from complex interactions between plant and soil within the rhizosphere, and not only from the plant genotype. Solid phases controlling Fe solubility in soils, chemical speciation of Fe in solution, importance of redox state in the solubilization of Fe, and the role of synthetic and natural chelates in transport process that occur near roots are among soil dependent factors determining Fe bioavailaibility (Lindsay, 1995). In addition, plant Fe uptake mechanisms are intimately intricated with loading processes of other metals, some of them being potentially toxic for humans. Ferritin over accumulation in transgenic tobacco leaves leads to an illegitimate Fe sequestration. As a consequence, these transgenic plants behave as Fe deficient, and activate Fe transport systems as revealed by an increase in root ferric reductase activity, explaining why these plants have an increased Fe content (Van Wuytswinkel *et al.*, 1999). This Fe deficiency situation has been reported to be responsible for cadmium loading of plants, through activation of the IRT1 ferrous Fe transporter (Vert *et al.*, 2002). In grasses, phytosiderophores of the mugineic acid family are involved in root Fe(III) uptake; this system, activated under Fe deficiency conditions, is also able to transport zinc, copper, nickel, manganese and cadmium (Schaaf *et al.*, 2004).

Indeed, the influence of various soil conditions on the increase in leaf Fe content of various tobacco plant genotypes has been recently tested (Vansuyt *et al.*, 2000). One control transgenic tobacco and two transgenic tobaccos overexpressing ferritin in the plastids or in the cytoplasm, respectively, were grown on five different soils, two of them being sewage sludge amended.

Although a significant increase in leaf Fe concentration was measured in transgenic plants overexpressing ferritin grown on 3 out of 5 soils, this increase was not a general rule. On some soils, leaf Fe concentration of control plants was as high as in transgenic plants grown on other soils. In addition, an increased phosphorus concentration in two sewage sludge amended soils correlated with a high leaf Fe concentration in control plants, similar to the one measured in ferritin overexpressing plants. Growing plants *in vitro* with various increasing phosphate concentrations revealed a direct role of P in Fe loading of control plants, at a similar level than overexpressing ferritin plants in standard P conditions. Additionally, with one of the soils tested, not only Fe but also manganese, zinc and cadmium, and to a much lesser extent copper, nickel and lead were found to be more abundant in ferritin overexpressing plants than in control plants. These data indicate that the Fe fortification of leaves, based on ferritin over expression, could be limited in its biotechnological application because of its high soil dependence.

7. CONCLUSIONS

In the last decade, tremendous progress have been made concerning the molecular characterization of the primary targets, such as transporters and ferritins, involved in Fe accumulation in the various organs of a plant during its growth and development. Transfer of this knowledge to biotechnological approaches aiming to improve the Fe content of crops has already started, opening promising opportunities to improve the Fe diet. To be fully valid, the Fe biofortification concept will need further inter-disciplinary studies integrating research from molecular plant physiology, animal and human nutrition and soil sciences.

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Chapter 18

METABOLIC CHANGES IN IRON-STRESSED DICOTYLEDONOUS PLANTS

GRAZIANO ZOCCHI
Department of Crop Production – Section of Plant Physiology and Biochemistry. University of Milano, Milano, Italy. E-mail: graziano.zocchi@unimi.it

Abstract: The response to iron deficiency stress in dicotyledonous plants is by far more complex than the simple activation of the reduction-based mechanism. In most of the Strategy I plants studied so far there is an associated increase in the activity of a plasma membrane H^+-ATPase which actively extrudes the protons necessary both for decreasing the rhizospheric pH and generating the electrochemical proton gradient needed to drive ion uptake. Along with these activities, localised on the root plasma membrane, it has been found that metabolism is involved in sustaining the production of reducing equivalents [NAD(P)H] and ATP. Many processes (glycolysis, respiration, etc.) are increased to efficiently sustain the response to iron depletion. In particular, the activity of phosphoenolpyruvate carboxylase is increased several fold. These increases could be linked to the production of substrates for the ferric chelate reductases and H^+-ATPase activities, generation of H^+ for the cytosolic pH-stat and organic acids. The activation of all these processes makes plants more efficient in the acquisition of iron.

Key words: ferric chelate reductase; FC-R; H^+-ATPase; organic acid; PEPC

1. INTRODUCTION

In the last few years we have seen the commencement of the biochemical characterisation of components of the Strategy I mechanism for iron uptake in plants. Since in plants employing this Strategy iron acquisition is mediated by a reduction-based mechanism, particular attention has been paid to the reduction of Fe^{3+} to Fe^{2+}. Recently, ferric chelate reductases (FC-R) have been cloned from *Arabidopsis*, pea and tomato (Robinson *et al.*, 1999; Waters *et al.*, 2002; Li *et al.*, 2004). A second breakthrough in elucidating

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the iron uptake mechanism was the successful cloning of the iron transporter protein IRT1 from *Arabidopsis* (Eide *et al.*, 1996) and later from pea (Cohen *et al.*, 1998) and tomato (Eckhardt *et al.*, 2001) roots. A review of these two topics is beyond the scope of this chapter and will be developed by others in this book.

The response to iron deficiency stress in dicotyledonous plants is more complex than the simple activation of the two-step mechanism considered above. In most of the Strategy I plants studied so far there is an associated increase in the activity of a plasma membrane H^+-ATPase, which actively extrudes the protons necessary both for decreasing rhizospheric pH and generating the electrochemical proton gradient needed to drive ion uptake (Rabotti and Zocchi, 1994; Dell'Orto *et al.*, 2000). Other biochemical activities are increased to efficiently sustain the response to iron depletion. Some of these, such as the synthesis of phenols and flavins are lineage specific responses. In this chapter I will focus on the metabolic changes which follow primary events and which are necessary to sustain the enzymatic activities involved in iron uptake.

2. GENERAL METABOLISM

The activation of reduction processes and the enhanced extrusion of protons under conditions of iron deficiency necessitate an increased rate of NAD(P)H and ATP regeneration relative to non iron stressed conditions. It has been shown that the NADPH:$NADP^+$ ratio (Sijmons *et al.*, 1984; Schmidt and Schuck, 1996) and ATP concentrations were higher under iron deficiency (López-Millán *et al.*, 2000; Espen *et al.*, 2000) and that in bean (Sijmons *et al.*, 1984) and *Plantago* roots (Schmidt and Schuck, 1996) the NADH/NAD^+ and NADPH/$NADP^+$ redox couples became more oxidised upon addition of ferric compounds. Under aerobic conditions, recharging of such substrates requires the acceleration of metabolism strictly linked to the respiratory pathway. The rate of O_2 uptake was increased under iron deficiency (López-Millán *et al.*, 2000; Espen *et al.*, 2000) although it is difficult to ascribe this major O_2 utilisation solely to the oxidative electron transport chain, since alternative oxidative pathways were also present (López-Millán *et al.*, 2000). In addition, O_2 could be directly used by the FC-R itself when plants were grown in the complete absence of iron. In this case, formation of H_2O_2 or other ROS compounds could result from the activity of the FC-R. Under iron deficiency some of the enzymes involved in detoxification of these compounds (i.e., peroxidase activity) were found to be enhanced in cucumber (Rabotti and Zocchi, 1994) and in sugar beet roots, where the activities of ascorbate-glutathione cycle enzymes were shown to

be increased (Zaharieva and Abadía, 2003). However, electron microscopy observation of apical root segments showed that in sections of iron deficient plants, the number of mitochondria was increased (Landsberg, 1994; Dell'Orto *et al.*, 2002). Microarray analysis of iron deficiency inducible genes revealed that in *Arabidopsis* there was an induction of components of the mitochondrial electron transfer chain, mainly cytocrome c reductase and oxidase (Thimm *et al.*, 2001) supporting the hypothesis of a respiratory control.

Surprisingly, in conditions where iron but not O_2 is limiting, enzymes associated with anaerobic metabolism have been shown to be upregulated (López-Millán *et al.*, 2000). This finding has been supported by microarray analysis (Thimm *et al.*, 2001), which showed induction of lactate dehydrogenase, pyruvate decarboxylase and alcohol dehydrogenase. These results might be interpreted as a by-pass system to maintain carbon flow and energy production by glycolytic reactions when, for some reason, O_2 become the limiting factor. In the absence of iron, heme synthesis should be impaired and the *de novo* synthesis of iron-containing proteins (i.e. cytochromes, Fe-S clusters) should not occur in mitochondria of iron deficient plants, despite the increased respiratory rate. This scenario has been shown to occur in the mitochondria of sycamore cells (Pascal and Douce, 1993), where the levels of iron containing components were greatly reduced. However, from substrate oxidation measurements, the decrease in these components seemed only to affect the functioning of complex II, while, apparently, neither complexes I, III and IV, nor the complex involved in the ATP synthesis were impaired in iron-deficient mitochondria. In fact, the calculated respiratory control and the ADP:O ratios did not show any difference between iron-sufficient and iron-deficient mitochondria, when complex II was not involved in O_2 consumption. However, mitochondria from iron-deficient cells displayed a more dilute matrix and most of the cristae were no longer evident. This is consistent with the marked decline in cytochromes and Fe-S clusters of iron deficient mitochondria that could not be incorporated into the inner membrane. These contrasting examples illustrate the complexity of the metabolic responses to iron deficiency.

3. CARBOHYDRATE METABOLISM

Glucose is the primary source for the synthesis of energetic substrates and for carbon skeleton supply in plant roots. In a metabolically active tissue, the elevated requirement of glucose is maintained by translocation of photosynthate from the shoot or by degrading starch stores in the roots. This has been shown to occur, for example, in bean plants (DeVos *et al.*, 1986)

where in the phloem sap of iron-deficient plants the sugar concentration doubled with respect to the control. Accordingly, microarray analysis showed that in shoots of *Arabidopsis* genes encoding α-amylase, the P_i translocator and the H^+-sucrose symporter were induced after three days of iron starvation (Thimm *et al.*, 2001). Carbohydrate concentration, (particularly glucose 6-phosphate and to a lesser extent fructose 6-phosphate), has been reported to increase in iron-deficient cucumber roots while the quantity of starch was greatly decreased (Espen *et al.*, 2000). The rate of carbohydrate catabolism was also increased under these conditions and several glycolytic enzymes have been shown to be activated (Sijmons and Bienfait, 1983; Rabotti *et al.*, 1995; Espen *et al.*, 2000) while increased levels of the glyceraldehyde 3-phosphate dehydrogenase protein were observed (Herbik *et al.*, 1996). These responses are likely associated with enhanced gene expression (Thimm *et al.*, 2001). In contrast, components of the carbohydrate biosynthetic pathway (for instance UDP-glucose) decreased, suggesting a general shift from anabolic to catabolic metabolism. Upregulation of glycolysis provides: i) ATP synthesis to sustain the major activity of the H^+-ATPase; ii) formation of reducing equivalents for the FC-R; iii) formation of phosphoenolpyruvate (PEP), iv) a contribution to the regulation of cytosolic pH (see below). Furthermore, other metabolic pathways and free cytosolic enzymatic activities are upregulated during iron deficiency. In particular, some cytosolic $NAD(P)^+$-dependent dehydrogenases such as glucose 6-phosphate dehydrogenase (oxidative pentose phosphate pathway) (Sijmons and Bienfait, 1983; Rabotti *et al.*, 1995; López-Millán *et al.*, 2000), malic enzyme (Sijmons and Bienfait, 1983; Rabotti *et al.*, 1995), glyceraldehyde 3-phosphate dehydrogenase (Sijmons and Bienfait, 1983; Rabotti *et al.*, 1995; Espen *et al.*, 2000), and isocitrate dehydrogenase (López-Millán *et al.*, 2000) showed increased activity in order to produce reducing equivalents necessary to keep the FC-R working at the necessary rate. Microarray data presented by Thimm *et al.* (2001) partially agree with conclusions from biochemical analyses. However, in *Arabidopsis*, microarray studies revealed changes in expression levels of only a few of the above-mentioned enzymes during iron deficiency. This does not necessarily contrast with the biochemical data obtained in other plant species; in fact, cucumber roots, for example, behave for some responses quite differently from *Arabidopsis* used in that study and many responses to environmental changes are likely to be mediated at the post-translational level and thus not detectable at the level of transcription. The importance of rhizosphere acidification in the responses of Strategy I plants to iron deficiency has been recognised for many years (Römheld and Marschner, 1986). This response has been associated with the activity of a plasma membrane localised H^+-ATPase (Zocchi and Cocucci, 1990).

However, unlike the FC-R, increased H^+-ATPase activity was not frequently observed and the extent of stimulation of the enzyme activity seemed to differ considerably among plant species and genotypes, ranging from a very low value to about 100% (Schmidt, 1999). In cucumber roots, one of the most active acidifying species, it has been shown by blotting analysis that the development of a higher activity under iron deficiency was associated with an increase in the level of the enzyme (Dell'Orto *et al.*, 2000) and of its transcript (De Nisi *et al.*, 2004). In the literature only one report (Yi and Guerinot, 1996) clearly showed acidification under iron deficiency by *Arabidopsis* roots, while for cucumber and other plant species acidification is a normal component of the deficiency response and is greatly enhanced. This could explain why in *Arabidopsis* Thimm *et al.* (2001) did not find any change in the expression of the H^+-ATPase. One could therefore hypothesise that, beyond the fundamental step of iron reduction, the efficiency responses rely on metabolic reactions that could be different in different species.

4. PHOSPHENOLPYRUVATE CARBOXYLASE

Among the metabolic activities that are increased under iron deficiency, phosphoenolpyruvate carboxylase (PEPC) deserves a separate and particular mention, since in many cases its activation is comparable, if not greater, than that of the primary responses. Its importance in plant metabolism is pivotal and in roots its anaplerotic features are unique. PEPC is a ubiquitous enzyme in plants which catalyses the fixation of bicarbonate to phosphoenolpyruvate to produce oxaloacetate and P_i. In C_3 plants and in non-photosynthetic tissues PEPC is active in several anaplerotic reactions to replenish tricarboxylic acid cycle intermediates, to provide carbon skeletons to sustain the synthesis of amino acids during NH_4^+ assimilation and for the regulation of cytoplasmic pH. In iron-deficient cucumber roots (De Nisi and Zocchi, 2000) and in the yellow zone of iron-deficient sugar beet roots (López-Millán *et al*., 2000) PEPC activity was, respectively, four and sixty times higher than in control roots. Besides the fact that PEPC is a highly regulated enzyme, both allosterically and covalently (Chollet *et al.*, 1996), increased activity is also attributable to increases in protein expression (as shown by immunoblotting analysis) (De Nisi and Zocchi, 2000; Andaluz *et al.*, 2002) and by induction of mRNA transcription (De Nisi *et al.*, 2004). Furthermore, it was shown that this increase was localised mainly to the external layers of the cortical cells of iron deprived root apical sections which are very active in proton extrusion (De Nisi *et al.*, 2002). As a consequence of increased proton extrusion and organic acid synthesis, an interesting correlation was shown between iron deficiency and PEPC activity (Landsberg, 1986;

Bienfait *et al.*, 1989; Rabotti *et al.*, 1995) and several working hypotheses have been proposed to explain this correlation (Landsberg, 1986; De Vos *et al.*, 1986; Espen *et al.*, 2000; López-Millán *et al.*, 2000). A first hypothesis follows the pH-stat theory formulated by Davies (1973), in which cytoplasm alkalinisation associated with proton extrusion would in turn activate PEPC activity to produce organic acids. This activation was interpreted as a major effort of the biochemical pH-stat system to re-equilibrate the cytosolic pH to its physiological value. This speculation was in part confirmed by an *in vivo* ^{31}P-NMR study which showed that the pH_c was effectively increased under Fe deficiency (Espen *et al.*, 2000). A perhaps more intriguing, yet related hypothesis suggested a more complex role for PEPC. Activation of proton efflux, as one of the main responses to iron deficiency, requires not only ATP production - to fuel the H^+-ATPase -, but also for H^+ to be extruded by the enzyme. Glycolysis could be seen as the pathway capable of satisfying both of these requirements. In fact, three glycolytic reactions are considered protogenic: those catalysed by hexokinase, phosphofructokinase (PFK) and glyceraldehyde 3-phosphate dehydrogenase (Sakano, 1998). To keep the rate of glycolysis sufficiently high, PEP must also be consumed at a high rate, as well. Its utilisation can be carried out by pyruvate kinase and/or by PEPC. Consumption of PEP relieves its own allosteric inhibition on the key glycolytic enzyme PFK. Furthermore, the PEPC activity increases P_i thus activating PFK by relieving the inhibition by PEP. In plants where the H^+-ATPase activity is high (i.e., cucumber roots), the activity of the PEPC has been shown to be very high, and as a consequence the rate of glycolysis is increased. After five days of iron deficiency, glyceraldehyde 3-phosphate dehydrogenase and phosphor-fructokinase activities were increased by 128% and 70%, respectively (Espen *et al.*, 2000).

The advantage of PEPC over PK in consuming PEP is not attributable only to favourable thermodynamics, but also to its ability to produce organic acids, which are protogenic (Davies, 1973). Moreover, PEPC activity could by-pass a possible negative control exerted by citrate over pyruvate kinase. This model assumes that proton extrusion is the driving downstream force for glycolysis and that PEPC is activated as a consequence of PEP accumulation. On the other hand, activation of PEPC leading to an increase in the rate of glycolysis would produce cytosolic acidification that would in turn activate the H^+-ATPase. Either of these scenarios would result in similar outcomes (Figure 18-1). There is another important feature in the activation of PEPC, the production of organic acids. It has been shown that under iron deficiency, plants often produce substantial amounts of organic acids, particularly malate and citrate (Abadía *et al.*, 2002 and references therein). We can assign two important roles to these organic acids: a chemical one and a biochemical one. The former will accomplish chelation and the

transport of iron outside (as exudates) and within the plant (vascular transport). The latter, much more complex, involves several aspects of metabolism.

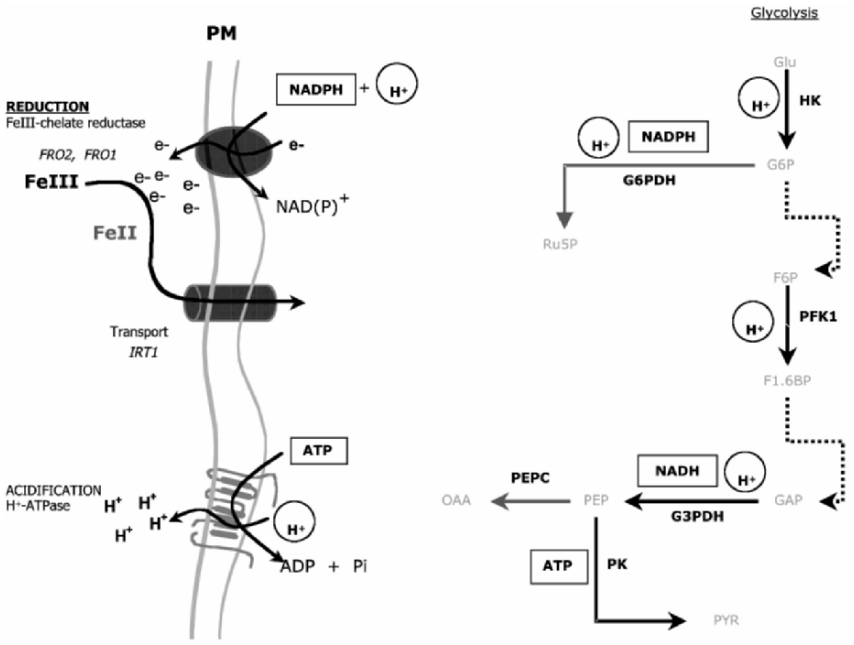

Figure 18-1. Interactions between membrane activities and carbohydrate metabolism are indicated by compounds enclosed in boxes and circles.

At least two points deserve attention. As stated above, production of organic acids is protogenic, and might thus be useful in controlling the cytosolic pH or to feed the increased activity of the PM H^+-ATPase. Second, elevated production of oxaloacetate and/or malate could be used to replenish the Krebs cycle. This anaplerotic function is important to keep the cycle working in the absence of or at limiting concentrations of pyruvate and to produce both ATP and citrate. These can be exported from mitochondria to accomplish both the chemical and biochemical cytosolic functions. In fact, they can be extruded from the roots to facilitate acquisition of iron from the soil or transported to the shoot via the xylem (Figure 18-2). Abadía and co-workers have hypothesised a role for citrate in providing carbon skeletons to chlorotic leaves to sustain growth and respiration (Abadía *et al.*, 2002). On the other hand, citrate could be used to produce reducing equivalents for the FC-R through the action of the cytosolic $NADP^+$-dependent isocitrate dehydrogenase and production of 2-oxoglutarate. Since it has been shown that protein synthesis is increased (see below) under iron deficiency and that

this requires synthesis of amino acids, 2-oxoglutarate and the same product of PEPC, oxaloacetate, could be used in nitrogen assimilation thus linking the metabolism of carbon with that of nitrogen.

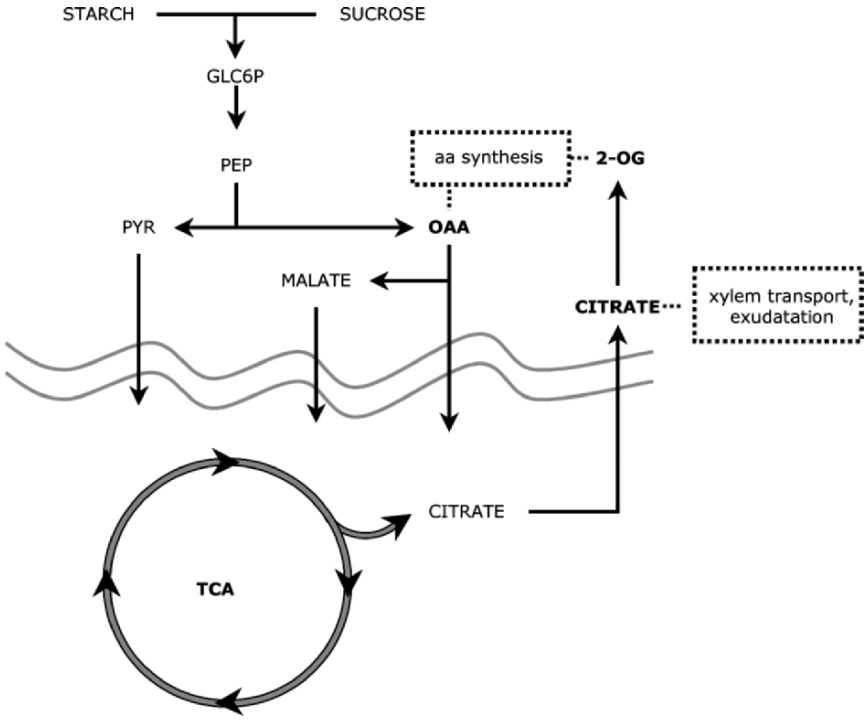

Figure 18-2. Metabolic model for organic acid utilisation.

5. PROTEIN SYNTHESIS

Responses to iron deficiency involve gene expression; a *de novo* synthesis of mRNAs and a general increase in the cellular protein content (Sijmons and Bienfait, 1983; Pontiggia *et al.*, 2003). Several works investigating iron deficiency responses have shown that the activities of a number of enzymes are increased and that this increment often corresponds to an increase in the protein content in addition to possible enzymatic regulation (Schmidt and Buckhout, 1997; Pontiggia *et al.*, 2003). This has been demonstrated for IRT1, PEPC and PM-H^+-ATPase by western blot analysis (Dell'Orto et al, 2000; De Nisi and Zocchi, 2000; Connolly *et al.*, 2002; Vert *et al.*, 2003). Similar increases in enzymatic activities linked to organic acid metabolism (López-Millán *et al.*, 2000) as well as to anaerobic

(Herbik *et al.*, 1996; López-Millán *et al.*, 2000) and carbohydrate (Sijmons and Bienfait, 1983; Rabotti *et al.*, 1995; Espen *et al.*, 2000) metabolism have also been demonstrated. Moreover, Schmidt (1999) showed that *de novo* synthesis of protein was required for the increased activity of the FC-R, by using the protein synthesis inhibitor cycloheximide. Furthermore, as a confirmation of this result, it has been shown that in swollen root tips, rhizodermal transfer cells have a high proportion of dense, organelle-rich cytoplasm as determined by electron microscopy analysis (Landsberg, 1994; Dell'Orto *et al.*, 2002; Pontiggia *et al.*, 2003). There is also clear evidence of major biosynthetic activity as the amount of polysomes extracted from iron-deficient roots is almost double that from controls (Pontiggia *et al.*, 2003). Furthermore, ^{14}C-uridine (synthesis of RNAs) and ^{14}C-leucine (protein synthesis) incorporation levels are higher in iron-deficient apical roots (Pontiggia *et al.*, 2003). Moreover, the amount of total amino acids was also increased in order to sustain the major protein synthesis occurring at this stage. In particular the concentrations of glutamate and aspartate were increased by 176% and 45%, respectively, as determined in cucumber roots (Pontiggia *et al.*, 2003).

6. CONCLUDING REMARKS

From this brief consideration of the metabolic changes induced in Strategy I plants by iron deficiency, we can draw some conclusion and future insights on metal nutrition. It appears that iron acquisition under limiting conditions is not simply linked to the induction of the FC-R and of high affinity transporters. The efficiency of a plant response to iron deprivation in its natural environment should be determined by its capacity to activate several mechanisms, which as a whole contribute to solving the problem. The more pronounced are the responses, the more efficient is the plant. It becomes clear that metabolism is deeply involved in the response to iron deficiency, with particular regard to the central role played by PEPC. In this chapter I have presented models to offer mechanistic explanations to the metabolic events which are involved in the response to iron deficiency in Strategy I plants. However, we are still far from gaining a full comprehension of the biochemical mechanisms involved. The new tools offered by molecular biology and the use of new mutants will be helpful in the elucidation of the key regulatory points, as they have been for our understanding of the FC-R and for iron transporters.

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Chapter 19

PROTEOMIC STUDIES UNDER IRON STRESS: IRON DEFICIENCY-INDUCED REGULATION OF PROTEIN SYNTHESIS IN THE GREEN ALGA *CHLAMYDOMONAS REINHARDTII*

IMMO REINHARDT[1], SOPHIE HAEBEL[2], ALEXANDRA HERBIK[1] and THOMAS J. BUCKHOUT[1]

[1]Applied Botany, Humboldt University Berlin, Invalidenstraße 42, 10115 Berlin, Germany. E-mail (T. J. B.): h1131dqy@rz.hu-berlin.de. [2]Center for Biopolymers, Potsdam University, Karl-Liebknecht-Strasse 24-25, 14476 Golm, Germany

Abstract: Iron assimilation in the unicellular green algae *Chlamydomonas reinhardtii* involves a reductive step. Using electrophoretic separation and mass spectrometry analyses, we have identified polypeptides whose synthesis and/or degradation were altered under Fe deficiency stress. Increases in O_2 evolving enhancer subunits (OEE) and ferritin were interpreted in terms of an altered structure of the photosynthetic apparatus and release of Fe. Two primary metabolic enzymes, namely PEP carboxykinase and isocitrate lyase, were also increased under Fe deficiency. These increases were interpreted as a re-direction of C from lipid stores to glucose synthesis, which may have resulted from decreased C fixation in photosynthesis. Plasma membrane proteins altered under Fe starvation were frequently of unknown function. In some cases the expression of the corresponding genes to these unknown proteins was regulated by Fe in similar manner to FLP (*Fox1*), a gene encoding the ferroxidase, which was known to participate in high-affinity Fe uptake. Most importantly, a membrane protein was identified that showed distinct sequence homology to Fe^{3+}-chelate reductases. Based on these findings a model has been proposed for the mechanism of Fe uptake at the *Chlamydomonas* plasma membrane.

Key words: *Chlamydomonas*; iron deficiency; plasma membrane; proteomics

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1. INTRODUCTION

With the increase in the atmospheric O_2 concentration approximately 2 billion years ago, the biosphere underwent a transition from a reducing to an oxidizing environment. As a consequence, the equilibrium concentrations of metals were shifted to the more oxidized species with dramatic changes in availability of some ions. For example, following oxidation, the availability of Cu was increased since at near neutral pH, Cu^{2+} is more soluble than Cu^{+}. However, following oxidation of Fe^{2+}, the availability of Fe was dramatically decreased. In contrast to Fe^{2+}, Fe^{3+} is a strong Lewis acid and at near neutral and alkaline pH, forms Fe^{3+}-hydroxides that polymerize into an amorphous, highly insoluble Fe hydroxide colloid. Furthermore, when Fe^{3+} is present in neutral solution, it is frequently present as chelated complexes that are relatively inert to ion exchange. Fe^{2+} also forms complexes with a variety of ligands, but because of its smaller cationic charge, these are usually less stable than with Fe^{3+}. It is the combination of these physical properties that result in the low availability of Fe in aerobic environments (Greenwood and Earnshaw, 1998).

Organisms including *Chlamydomonas* have developed mechanisms to acquire Fe and regulate its concentration in the cell. In this article, we will report on our findings regarding the mechanism of Fe uptake in *Chlamydomonas* and show original data documenting changes in the cytosolic and plasma membrane proteome that occur in response to Fe deficiency.

1.1 Mechanisms involved in iron uptake

Iron can catalyze a large number of both enzymatic and non-enzymatic oxidation-reduction reactions in the cell. Non-enzymatic reactions, which are catalyzed principally by Fe^{2+}, generate free radicals and other reactive O_2 species (ROS). These lead to oxidative stress and cell death. All aerobic organisms must balance the requirement for Fe against the danger of Fe toxicity and have, therefore, developed high-affinity Fe transport pathways that are regulated by the concentration of Fe or Fe-containing complexes in the cell. In addition to the high-affinity Fe transport pathways, a large number if not all organisms have other low-affinity transport systems that catalyze Fe uptake at Fe concentrations at which the high-affinity system is saturated. In the past two decades much information has been obtained in eukaryotes with regard to the mechanisms of Fe uptake and the regulation of the Fe concentration in the cell. In general, there are two mechanisms by which Fe is transported at the cell surface. In the first case, Fe is taken up following reduction of an Fe^{3+}-chelate. Fe^{2+}, which is released from the

chelate, is either transported directly or is re-oxidized by a ferroxidase to Fe^{3+} prior to transport. The second mechanism involves siderophores, which are synthesized and secreted into the apoplast under conditions of Fe deficiency. Siderophores form Fe^{3+}-siderophore complexes by exchange with the chelated Fe^{3+} present in the soil. The siderophore-chelated Fe is subsequently taken up into the cell. Both mechanisms are widely found in nature; however, in this report, only the reductive mechanism for Fe uptake will be discussed.

1.2 High-affinity iron uptake in yeast

The mechanism of Fe uptake in fungi has been most frequently studied in *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Candida albicans*. However, the mechanism of Fe uptake in *Saccharomyces* has been studied in most detail and because of this and other reasons that will become apparent, regulation of Fe uptake in *Saccharomyces* will be briefly summarized. Lesuisse and Labbe (1989) were the first to recognize that reduction of extracellular Fe^{3+} was a prerequisite for high-affinity Fe uptake in *Saccharomyces*. Subsequently, Dancis *et al.* (1990) and Georgatsou and Alexandraki (1994) cloned the genes, *FRE1* and *FRE2*, encoding Fe^{3+}-chelate reductases that were shown to be essential for high-affinity Fe uptake. Surprisingly at that time, Dancis *et al.* (1994) reported that deletion of the high-affinity Cu transporter, Ctr1p, led to loss of high-affinity Fe uptake. The dependence of high-affinity Fe uptake on copper nutrition had been long before recognized in swine that showed Fe deficiency when fed on a copper deficient diet (Lee *et al.*, 1968).

In a fascinating series of publications (reviewed by Askwith *et al.*, 1996), the molecular basis for the link between copper nutrition and Fe uptake was established. It is now known that high-affinity Fe transport in yeast depends upon a copper-containing protein, Fet3p (Askwith *et al.*, 1994). The *FET3* gene is essential for high-affinity Fe uptake and its deletion or mutation results in Fe deficiency under normal Fe supply. In the yeast plasma membrane, the multicopper oxidase, encoded by *FET3*, and an Fe^{3+} transporter, encoded by *FTR1*, are both required for high-affinity Fe uptake (Stearman *et al.*, 1996). Proper expression of *FET3* is required for the transport of FTR1p to the plasma membrane. Thus, high-affinity Fe transport in yeast involves first the reduction of Fe^{3+} followed by the re-oxidation of Fe^{2+} to Fe^{3+}, which is then transported into the cell. The *FRE1*/*FET3*/*FTR1* transport system is regulated by the Fe transcription factors, Aft1p and Aft2p (Blaiseau *et al.*, 2001). During Fe deficiency, these transcription factors activate high-affinity Fe uptake. The combination of reduction, re-oxidation

and transport is energy-demanding but has the potential advantage of imparting specificity and selectivity to the transport system.

In yeast, low-affinity Fe^{2+} transport required the *FET4* gene (Dix *et al.*, 1994). *FET4* encodes a protein that catalyzed the transport of Fe^{2+} among other divalent metals. This transport system was independent of reductase-oxidase mentioned above, was not regulated by Aft1p and did not require O_2 (Jensen and Culotta, 2002). In addition to Fet4p, a second group of divalent cation transporters, the Nramp (natural resistance-associated macrophage proteins) family, has been shown to contribute to transport of several metals including Fe. In *Saccharomyces cerevisiae* 3 *Nramp* genes have been identified (*SMF1*, *SMF2* and *SMF3*; Portnoy *et al.*, 2000). *SMF1* and *SMF2* were specific for Mn^{2+}, whereas *SMF3*, which was expressed in the vacuole, was believed to be involved in regulation of the vacuolar Fe concentration and was repressed under sufficient Fe (Portnoy *et al.*, 2000). At present, the *FET4* system is thought to be the primary low-affinity Fe transport system in yeast. The role that Nramp transporters may play in Fe transport remains to be clarified.

1.3 Iron uptake in *Dunaliella*

The halotolerant unicellular green algae, *Dunaliella salina*, is capable of surviving in water nearly saturated with salt. Sadka *et al.* (1991) reported that a 150 kDa (p150) plasma membrane protein coincidently increased with rising salinity. Subsequently, Fisher *et al.* (1997) reported an increase in p150 also under Fe deficiency. Based on cDNA sequence similarity, the protein was identified as triplicated transferrin (TTF), a member of the transferrin family of proteins, which had been reported only in animals (Fisher *et al.*, 1997). Fisher *et al.* (1998) further showed that TTF accumulated to high levels under Fe deficiency and that this accumulation correlated with an enhanced Fe uptake. High salinity (e.g. 3.5 M NaCl) did not impair its activity, and Fe uptake was strictly dependent on bicarbonate. It was proposed that TTF acted in Fe uptake other than by receptor-mediated endocytosis and that its presence permitted the cells to overcome a possible limitation in Fe availability under high salinity (Fisher *et al.*, 1997). Although much remains to be learned with regard to the mechanism of Fe uptake in *Dunaliella*, current information points to a mechanism that is unique in microorganisms and significantly different to that found in yeast and higher plants.

1.4 Iron homeostasis in *Chlamydomonas*

Recent advances have been made with regard to elucidation of the Fe uptake mechanism in *Chlamydomonas*. In particular, the availability of numerous EST (expressed sequence tags; Shrager *et al.*, 2003; www. chlamy.org/search.html) and the completion of the genome sequence (genome.jgi-psf.org/chlre2/chlre2.home. html) have contributed greatly to this progress. In *silico* analysis of the EST database resulted in the identification of a number of sequences that showed significant similarity to *Saccharomyces* genes and encoded proteins involved in high-affinity Fe uptake (La Fontaine *et al.*, 2002). Among the genes identified there was a putative multicopper ferroxidase [*Fox1*, which is synonymous with FLP (Herbik *et al.*, 2002b)], a putative Fe transporter (*Ftr1*), two Cu-ATPases (Ccc2), a copper chaperone (*Atx1*) and the Fe storage protein ferritin (*Fer1*). *Fox1* (FLP) has significant homology to hephaestin and *FET3*, which both encode ferroxidases that are an integral part of the high-affinity Fe uptake in mammals and *Saccharomyces*, respectively. La Fontaine *et al.* (2002) could show that the expression of both *Fox1* and *Ftr1* were induced under Fe-deficient growth conditions.

Physiological and biochemical evidence indicated that Fe uptake in *Chlamydomonas* resembled that found in *Saccharomyces*. In *Chlamydomonas*, an Fe^{3+}-chelate reductase and an Fe transport activity were induced in parallel in cells growth under Fe deficiency (Eckhardt and Buckhout, 1998; Lynnes *et al.*, 1998; Weger, 1999). More recently, a distinct increase of a ca. 150-kD protein on the plasma membrane with sequence homology to mammalian hephaestin and multicopper oxidase, FET3p, was observed during growth under Fe deficiency (Herbik *et al.*, 2002a). FLP (*Fox1*) was transcribed under conditions of Fe deficiency, and consistent with the role of a Cu-containing protein in Fe transport, growth of cells in Cu-depleted media eliminated high-affinity Fe uptake. Furthermore, Cu-deficient cells that were grown in suboptimal Fe showed greatly reduced Fe accumulation compared with control, Cu-sufficient cells. Based on these observations, FLP (Fox1p) was suggested to be a component of the Fe uptake complex with a function analogous to FET3p in *Saccharomyces*.

In addition to the high-affinity Fe transport system, there are several genes in the *Chlamydomonas* genome that may be involved in low-affinity Fe transport. Only few of these potential transporters have been characterized. A *Chlamydomonas* homologue to *DMT1*, an Nramp homologue in humans that is proposed to transport Fe in the duodenum (Rosakis and Köster, 2004), was shown by complementation in yeast to have a broad substrate specificity, catalyzing transport of Mn^{2+}, Fe^{2+}, Cd^{2+} and Cu^{2+} but not Zn^{2+} (Rosakis, 2003). Although *DMT1* from *Chlamydomonas* catalyzed

low-affinity Fe transport in yeast, the identity of the low-affinity Fe transport system in *Chlamydomonas* has not been conclusively established.

In an attempt to identify further components involved in Fe uptake and assimilation in *Chlamydomonas*, we have investigated changes in the cytosolic and plasma membrane proteome during adaptation to Fe deficiency stress. We have used 2-dimensional gel electrophoresis to visualize changes in proteome composition and mass spectrometry to identify these polypeptides when possible. The results of these investigations are presented below.

2. MATERIALS AND METHODS

2.1 Strain and culture conditions

The *Chlamydomonas reinhardtii* cell wall-deficient mutant strain 83.81 was obtained from the *Sammlung von Algenkulturen*, Göttingen, Germany. Cells were grown mixotrophically at room temperature (22-24°C) in Fe-sufficient (ca. 18 μM) and Fe-deficient TAP medium (Harris, 1988) with moderate shaking (80-100 rpm) and constant illumination of about 100 μmol m^{-2} s^{-1} photosynthetic active radiation as described previously (Eckhardt and Buckhout, 1998). Cell density was estimated by measuring the absorbance at 750 nm (Harris, 1988). All glassware was rinsed with 0.1 N HCl. Chemicals of analytical purity were used.

2.2 Isolation of soluble and plasma membrane protein fractions

Cells from exponentially growing cultures were collected by centrifugation at 2,000 *g* for 3 min. Ten g of cells were used to prepare the soluble fraction. After re-suspension in extraction buffer (250 mM sucrose, 50 mM HEPES pH 7.5, 1 mM EGTA, 50 mM KCl, 0.6% w/v PVPP, 0.1 mM PMSF, 2.5 mM DTT), the cells were disrupted by sonication on ice (SONOPULS HD 2070, Sonotrode KE 76, Bandelin Co., Berlin, Germany, 2 x 5 min). Homogenates were differentially centrifuged (4°C and 9,800 *g* for 30 min) and the resulting supernatant re-centrifuged (SW 27 rotor, Beckman Instruments, Palo Alto, USA) for 1 h at 100,000 *g*. The resulting supernatant was defined as the "soluble fraction".

Plasma membranes were prepared using 2-phase partitioning as described in Larsson and Møller (1990) as modified by Norling *et al.* (1997). Cells (2 to 4 g fresh weight) were re-suspended in extraction buffer (see

above) and mechanically homogenized using a Polytron (KINEMATICA AG, Littau, Switzerland). Three differential centrifugations were performed (1,200 g for 3 min, 13,000 *g* for 5.5 min and 100,000 *g* for 1 h). The pellet from the last centrifugation was referred to as the "microsomal fraction" and was re-suspended in 250 mM sucrose, 5 mM K-phosphate pH 7.8, and 5 mM DTT. The polymer phases (dextran/polyethylene glycol, 6.5% w/w) contained 5 mM KCl. After 3 partitioning steps, the final upper phase was diluted in 0.33 M sucrose, 5 mM MOPS pH 7.0, 1 mM EDTA and 2 mM DTT, and the plasma membranes were collected by centrifugation (100,000 *g* for 1h) and re-suspended as appropriate. Plasma membranes were carbonate washed according to Fang *et al.* (1987). Two-hundred µl of membrane suspension were 10-fold diluted with 0.1 M Na_2CO_3 (pH 11.5), incubated on ice for 20 min and diluted with Na_2CO_3 to 30 ml. The plasma membranes were collected by centrifugation (100,000 *g* for 1 h) and re-suspended in medium as above.

Proteins from the soluble and plasma membrane fractions were obtained by phenolic extraction according to Meyer *et al.* (1988). One ml (approximately 1 mg protein) of the soluble or the plasma membrane fraction was incubated for 15 min with 5 ml Tris-saturated phenol (pH 7.0) at 4°C. Following centrifugation (15 min at 5,445 *g* and 4°C), the upper phase and the interphase were removed and discarded. The lower phase was re-extracted with an equal volume of Tris-phenol and centrifuged as above. The proteins from the lower phenolic phase were precipitated overnight at -20°C in 4 volumes of methanolic 0.1 M ammonium acetate. The resulting pellets were washed 3 times with ice-cold methanol and briefly dried to remove excess methanol.

2.3 Gel electrophoresis

For 1-dimensional PAGE (Laemmli, 1970), the protein pellets were dissolved in "loading buffer" (62.5 mM Tris-HCl pH 6.8, 2.5% (w/v) SDS, 10% (w/v) glycerol, 100 mM DTT) and heated for 3 min at 95°C. The protein concentration was determined using the method of Bradford (1976). Twenty µg of protein were loaded per lane. Following electrophoresis gels were stained with Coomassie R250 (soluble proteins) or with colloidal Coomassie in the case of plasma membrane proteins (Roti-Blue, Roth Co., Karlsruhe, Germany).

For 2-dimensional PAGE, the protein pellets were solubilized in a suitable volume of 7 M urea, 2 M thiourea, 3% (w/v) 3-([3-cholamidopropyl]-dimethylammonio)-1-propanesulphonate (CHAPS), 0.5% (v/v) Triton X-100, 40 mM dithiothreitol (DTT), 200 µM PMSF, 0.5% (v/v) IPG Buffer pH 3-10 NL (Amersham Biosciences, Freiburg, Germany) and incubated for 30

min at room temperature and 2 h at 4°C with shaking. Before gel loading the samples were centrifuged for 15 min at 20,600 *g* and 4°C. Up to 600 µg soluble proteins were used for a 13 cm nonlinear IPG strip (pH 3-10). Isoelectric focusing (IEF) was performed in the IPGphor chamber (Amersham Biosciences, Freiburg, Germany) using the following protocol: overnight strip rehydration in the sample solution at low voltage (11 h 30 V, 2 h 60 V), 1h 150 V, 30 min 500 V, 30 min 1000 V, 30 min 3000 V, 8 h 7000 V. A typical run was about 60 kVh. The temperature was 20°C.

After IEF the IPG strips were equilibrated for SDS-PAGE with 6 M urea, 30% (w/v) glycerol, 50 mM Tris-HCl pH 8.8, 5% (w/v) SDS and 50 mM DTT for 20 min. In a second equilibration step 143 mM iodoacetamide was used instead of DTT. The equilibrated strips were placed on top of a vertical SDS gel (12.5% acrylamide) and embedded in agarose. SDS-PAGE was run at 30 mA per gel in a HOEFER SE 600 apparatus (Amersham Biosciences, Freiburg, Germany). Following electrophoresis gels with soluble proteins were stained as above.

2.4 In-gel digestion, purification of peptides and MALDI-TOF MS

Polypeptides of interest were excised from the gels and de-stained for 2 h in a mixture of 40% acetonitrile and 60% (v/v) 50 mM NH_4HCO_3. De-stained gel pieces were dried by vacuum centrifugation. The dried gel fragments were digested by adding approximately 20 µl of a modified trypsin solution (30 ng/µl in 50 mM NH_4HCO_3; Roche Diagnostics, Mannheim, Germany). Subsequently, 30 µl of 50 mM NH_4HCO_3 were added and the digestion continued overnight at 37°C. Proteolytic peptides were extracted by incubation with approximately 15 µl of 5% formic acid for 10 min followed by the addition of approximately 30 µl of acetonitrile. The supernatant was removed after 30 min and the 5% formic acid/ acetonitrile treatment was repeated. The combined supernatants were lyophilized, dissolved in 20 µl 0.1% TFA and were then used for MALDI-TOF MS.

The peptides were separated by RP-HPLC (SMART system, Pharmacia, Uppsala, Sweden) on a Pharmacia C2/C18 SC 2.1/10 column using a linear acetonitrile gradient in 0.1% TFA. Peptides were detected at a wavelength of 214 nm. Mass spectra of peptide mixtures as well as post-source decay (PSD) spectra were obtained using a Reflex II MALDI-TOF mass spectrometer (Bruker-Daltonik, Bremen, Germany). α-cyano-4-hydroxy cinnamic acid [15 mg/mL in 7 vol acetonitrile and 3 vol 0.1% (v/v) TFA] served as the matrix. Samples were prepared by mixing 0.5 µl of 2%

aqueous TFA, 0.5 µl of sample and 0.3 µl of matrix solution directly on the target and air drying. For HPLC purified peptides the 2% aqueous TFA was omitted.

2.5 Western blot analysis of thylakoid membranes

Thylakoid membranes from *Chlamydomonas* cells were isolated according to Chua and Bennoun (1975) as modified by Fischer *et al.* (1997). In brief, cells were re-suspended in 25 mM Hepes-KOH pH 7.5, 5mM $MgCl_2$ and 0.3 M sucrose and homogenized using a Polytron. Thylakoid membranes were prepared by differential sucrose-gradient centrifugation and the fraction obtained were diluted in buffer and pelleted at 100,000 g for 1 h. Protein equivalent to 5 µg chlorophyll were solubilized in SDS loading buffer (see above) and were separated by SDS-PAGE on minigels (Bio-Rad Labs. GmbH, Munich, Germany). The proteins were blotted onto PVDF membranes (Fluorobind, Serva Feinbiochemica, Heidelberg, Germany) and Western blots were performed using polyclonal antibodies raised against 2 of the 3 peripheral subunits of the O_2 evolving complex (PsbO, PsbP) and PetB (cytochrome b_6). Antibodies were kindly provided by Dr. K.-D. Irrgang (Institute of Biophysical Chemistry, Technical University, Berlin). The PsbO antibody was directed against the protein isolated from oats, and the PsbP and PetB antibodies were raised against spinach proteins. The secondary antibody was conjugated to alkaline phosphatase. Protein bands were visualised using SIGMAFAST™ Fast Red TR/Naphthol (Sigma Chemical Co., Munich, Germany).

2.6 Northern blot analyses

Total RNA from *Chlamydomonas* was isolated as follows: approximately 1.1 x 10^7 cells were harvested by centrifugation (3 min, 2,300 *g* at 4°C) and immediately re-suspended in buffer (20 mM MES pH 7.0, 4 M guanidine-isothiocyanate, 20 mM EDTA and 50 mM mercaptoethanol). The samples were sonicated briefly, the suspension was extracted with phenol/chloroform/ isoamylalcohol (25:24:1), and the phases were separated by centrifugation (10 min at 13,000 *g* and 4°C). Acetic acid (1/20 volume of a 1 N solution) and 0.7 volumes of 100% ethanol were added to the upper phase. Samples were incubated for 1 h at –20°C and centrifuged (5 min at 13,000 *g* and 4°C). The resulting pellets were washed once with ethanol, once, in 200 µl 3 M sodium acetate and re-suspended in 200 µl RNase-free H_2O. Following addition of 0.1 volume of 6 M ammonium acetate and 3 volumes of ethanol and incubation for 30 min at –20°C, RNA was collected by centrifugation, washed with 70% ethanol and finally re-suspended in H_2O.

Twenty-five μg of total RNA were separated on an agarose gel and then blotted onto Hybond N+ membranes (Amersham Biosciences, Freiburg, Germany). Blots were hybridized with ^{32}P-dCTP-labelled fragments (Amersham Biosciences) of the 3 genes encoding the O_2 evolving enhancer proteins, OEE1-3 (PSBO, PSBP and PSBQ). The fragments were amplified by PCR from *Chlamydomonas* cDNA using the following primer combinations:

PsbO: f 5'-gattcagggcctgacctacc-3', r 5'-ggcagcggcaacgtttggcc-3';

PsbP: f 5'-gtccggcttcgtgccctacg-3', r 5'-gtaggcaccgtcggaaccgg-3';

PsbQ: f 5'-cctgttcgatgaccgctcgg-3', r 5'-cggcggccaggacggagtcc-3'.

Hybridization products were detected using HyperfilmTM MP (Amersham Biosciences).

3. RESULTS

3.1 Adaptation of the cytoplasmic proteome to iron deficiency

Chlamydomonas was cultured in continuous light at 22 ± 2°C in mid-log phase to a cell density of approximately 5-6 x 10^6 cells/ml and harvested by centrifugation. Cells were washed and re-suspended in either sufficient or deficient Fe media as appropriate. Following growth for 24 h, cells were harvested and the soluble proteins analyzed by 2-dimensional electrophoresis as described above. Electrophoresis conditions were optimized with regard to solubilization and resolution in the first and second dimensions. Approximately 650 distinct polypeptides were consistently resolved using this method (Figure 19-1). Of these approximately 650 polypeptides, surprisingly only 14 showed a consistent differential expression under Fe deficiency and only few of these could be clearly associated with a response to Fe assimilation or utilization (Figure 19-1 and Table 19-1).

3.1.1 Functional classification of cytoplasmic polypeptides

The polypeptides that were identified can be subdivided into groups with respect to their predicted functions (Table 19-1). The first category contains polypeptides presumably involved in regulation of translation. Eukaryotic translation and/or initiation factors are essential components for protein synthesis and polypeptide elongation. The translation/initiation factor elF-4A, the elongation factor elF-2 and elongation factor tufA were all consistently decreased under Fe deficiency. In contrast, the elongation factor

EF-1α was increased under the same conditions. Translational control of protein synthesis contributes to the response to changes in the environment. In plants translational regulation has been reported for water and heat stress, metabolic regulation and in the light to dark transition (Kawaguchi and Bailey-Serres, 2002). The changes in polypeptide synthesis and/or breakdown may indicate a translational regulation in response to Fe deficiency; although, from the present data, little can be said with regard to the nature of this regulation.

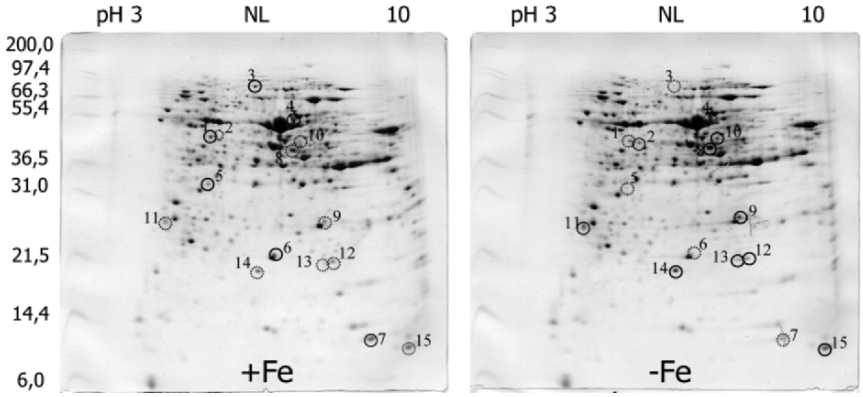

Figure 19-1. 2D-PAGE of soluble proteins from *Chlamydomonas* grown 24 h under iron-sufficient or -deficient conditions. Soluble proteins were prepared and separated as described in the Materials and Methods. Isoelectric focusing was performed using nonlinear pH gradients between pH 3 and 10. Separation in the second dimension was performed on 12.5% acrylamide gels. Circled polypeptides were differentially expressed under iron deficiency. The numbers correspond to the polypeptides listed in Table 19-1.

The second category of polypeptides was involved in metabolic reactions. In this group two polypeptides were identified: methionine synthase and isocitrate lyase. Genes encoding both of these proteins were induced in response to Fe deficiency in *Arabidopsis* (Buckhout and Thimm, 2003). In the case of *Arabidopsis*, it was speculated that the induction was in response to an increased activity of the glyoxylate cycle and metabolism of fatty acids under Fe deficiency and to an increased demand for methionine as a methyl donor and precursor for synthesis of secondary metabolites.

The third category contained the sole polypeptide that was clearly involved in Fe homeostasis, ferritin. Although it might seem paradoxical that ferritin, an Fe storage protein, was increased under Fe deficiency, the synthesis of ferritin may have been a result of the breakdown of chloroplast

proteins in response to Fe deficiency. As a result of breakdown in photosystem I, for example, large amounts of Fe would be released and could trigger increased ferritin synthesis. An increase in ferritin expression has been previously observed in *Chlamydomonas* in response to Fe deficiency (La Fontaine *et al.*, 2002).

Table 19-1. Summary of soluble proteins that were differentially synthesized in *Chlamydomonas* under iron deficiency. The soluble protein fraction was extracted with phenol and analyzed by 2-dimensional PAGE. Differentially expressed polypeptides were further analyzed by MALDI-TOF-MS. The response to iron deficiency (-Fe) is indicated as increased (+) or deceased (-). The molecular mass was estimated using standard proteins, and the pI was determined from the deduced protein sequence.

	-Fe	app. MW (kDa)	pI	Peptide sequences	Protein identity
1	-	46	5	RDELTLEGLK GLDVLQQAQSTGK	eukaryotic initiation factor elF-4A (*N. tabacum*)
2	+	45	5	GCELPLDQAVAR	methionine synthase (*C. reinhardii*)
3	-	85	6	GFVQFIYEPIK AYLPVVESFGFTSVLR	elongation factor elF-2 (*C. kessleri*)
5	-	33	5	peptide fragments	elongation factor tufA (*Synechococcus sp.* PCC 7002)
6	-	22	6.3	peptide fragments	OEE2 (*C. reinhardtii*)
7	-	12	8.5	EGFTQAR (N/L)(N/D)DLPQNVR FD(L/I)NT(L/I)ASTK	OEE3 (*C. reinhardtii*)
8	+	40	7	INNAFQR	isocitrate lyase (*C. reinhardtii*)
9	+	27	7.5	peptide fragments	elongation factor EF-1α (*A. thaliana*)
11	+	27	4	VSEYVSQLR	ferritin (*C. reinhardtii*)
12	+	21	7.5	peptide fragments	OEE2
13	+	21	7.6	peptide fragments	OEE2
14	+	20	6	peptide fragments	OEE2
15	+	10	9.5	peptide fragments	OEE2

The fourth and final category of soluble polypeptides whose synthesis and/or degradation was altered under Fe deficiency, contained polypeptides involved in photosynthesis. With regard to the discussion concerning ferritin, one might have expected an increase in a number of polypeptides associated with photosynthesis. This, however, was not the case. Of all polypeptides involved in photosynthesis, only the oxygen evolving enhancers (OEE) were detected. Polypeptides 12-15 (Table 19-1) were all increased under Fe deficiency, while 2 OEE polypeptides were decreased

(polypeptides 6 and 7, Table 19-1). The presence of OEE polypeptides was further investigated by Northern and Western blot analyses.

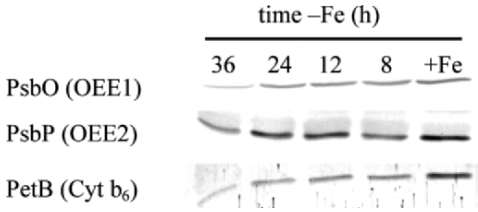

Figure 19-2. Western blot analysis of PsbO (OEE1) and PsbP (OEE2) in thylakoid membrane isolated from *Chlamydomonas* cells grown in the presence (+Fe) or absence of iron for 8, 12, 24 and 36 h. Thylakoid membranes were isolated as described and Western blots performed using polyclonal antibodies. The PsbO antibody was against the protein isolated from oats, the PsbP andPetB antibodies were raised against spinach proteins. The secondary antibody was conjugated to alkaline phosphatase.

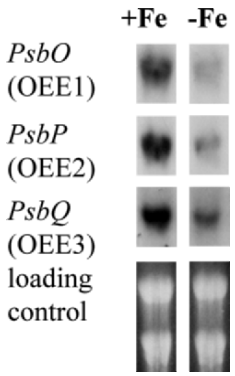

Figure 19-3. Northern blot analysis of *PsbO*, *PsbP* and *PsbQ* expression in iron-sufficient and -deficient *Chlamydomonas* cells. Twenty-five µg of total RNA were separated on an agarose gel and then blotted onto Hybond N+ membranes. Blots were hybridized with ^{32}P-dCTP-labelled fragments of the 3 genes encoding the O_2 evolving enhancer proteins, OEE1-3 (*PsbO*, *PsbP* and *PsbQ*). The fragments were amplified by PCR from *Chlamydomonas* cDNA using primer combinations described in the Materials and Methods section.

3.1.2 Western and Northern blot analyses of OEE polypeptides

The results observed for OEE polypeptides might have indicated that the photosynthetic apparatus was being degraded under Fe deficiency. This

would explain the increased presence of polypeptides 12 - 15 in solution. The fact that the OEE polypeptides 6 and 7 were decreased under Fe deficiency, could be the result of the partial degradation of OEE polypeptides. To confirm the changes in OEE polypeptides that were observed by gel electrophoresis, the presence of OEE polypeptides was analyzed immunologically using isolated thylakoid membranes and antibodies specific for OEE1, OEE2 and PetB (Figure 19-2). PetB, cytochrome b_6, was decrease already at 8 h and was nearly absent in thylakoid membranes after 36 h of Fe deficiency. The presence of OEE1 and OEE2, in contrast, remained fairly constant even after 24 h but was greatly decreased after 36 h Fe.

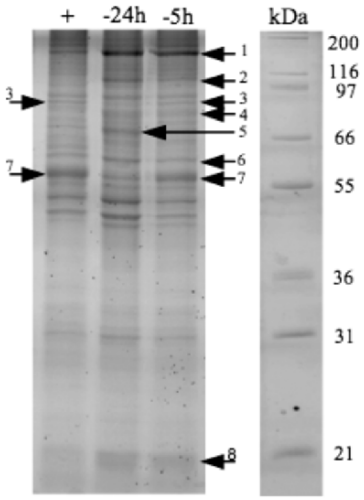

Figure 19-4. 1-dimensional PAGE of plasma membrane proteins from *Chlamydomonas* grown 5 and 24 h under iron-sufficient (+Fe) or -deficient (-Fe) conditions. Membrane proteins were prepared and separated as described in the Materials and Methods. Molecular mass markers are shown on the right. Arrows highlight polypeptides that were differentially expressed under iron deficiency. The number correspond to the polypeptides listed in Table 19-2.

To extend these results, Northern blot analysis was performed using gene-specific probes to each of the 3 *OEE* genes (Figure 19-3). Only one time-point after 24 h of Fe deficiency was investigated. In general, all 3 genes showed clearly decreased expression under Fe deficiency. Taken together with the results obtained from the Western blot and gel electrophoresis analyses, it appeared that significant breakdown and reduced synthesis of the photosynthetic apparatus occurred under Fe deficiency.

3.2 Adaptation of the plasma membrane proteome to iron deficiency

In parallel to the analysis of the adaptation of the cytoplasmic proteome to Fe deficiency, an investigation of changes in the plasma membrane proteome was undertaken. Plasma membranes were isolated by the 2-phase procedure and thoroughly washed with carbonate at pH 11.5 to remove contaminating soluble proteins. In general, the 2-phase method yielded a plasma membrane fraction with a purity of 85 to 90%. The major contaminating membranes were derived from the endoplasmic reticulum and Golgi apparatus; however, marker enzymes for these fractions have not been well established in *Chlamydomonas* (Buckhout, unpublished).

Following electrophoretic separation gels were analyzed visually for differentially expressed proteins. A typical 1-dimensional gel is shown in Figure 19-4. As was the case for soluble proteins, the presence of relatively few polypeptides was altered in response to Fe deficiency, and of these polypeptides, only a minority could be assigned a function based on sequence analysis. As is typical for all hydrophobic membrane proteins, analysis by MS was challenging. Nonetheless, it was possible to obtain sequences for polypeptides with hydrophobic amino acid sequences predicted to transmembrane domains. FLP, whose synthesis as been previously shown to be markedly increased under Fe deficiency, was also detected as a distinct band at approximately 150 kD. FLP was likely a largely soluble protein with a transmembrane domain on the N-terminus of the protein (Herbik *et al.*, 2002b, see also Discussion).

For 3 polypeptides in the plasma membrane fraction, whose synthesis or degradation were altered under Fe supply, no transmembrane domains were predicted (polypeptides 3, 5 and 7; Figure 19-4 and Table 19-2). The remaining 5 polypeptides contained at least 1 transmembrane domain. Polypeptides 3 and 5 were identified as heat shock proteins, one of which was increased and one decreased under Fe starvation. PEP carboxykinase (polypeptide 6, Table 19-2) was typically a cytosolic enzyme involved in gluconeogenesis. Although the available information is still limited, an increased synthesis of isocitrate lyase (Figure 19-1) and PEP carboxykinase (Figure 19-2) could indicate an increased activity of the glyoxylate cycle coupled with gluconeogenesis. This might be in response to decreased photosynthesis and/or respiration as a direct result of Fe deficiency.

Two polypeptides were of immediate interest with regard to Fe transport across the plasma membrane. FLP (Fox1p) has already been discussed. Polypeptide 2, migrating at approximately 100 kDa was increased and showed distinct homology to several Fe reductases, namely Fro2p, Fre1p and Frp1p. Investigation of the expression of the reductase genes in

Chlamydomonas is in progress. At least some of these genes were induced under Fe deficiency (Reinhardt and Buckhout, unpublished).

Table 19-2. Summary of plasma membrane polypeptides differentially synthesized in *Chlamydomonas* under iron deficiency. The plasma membrane protein fraction was extracted with phenol and analyzed by SDS-PAGE. Differentially expressed polypeptides were further analyzed by MALDI-TOF-MS. The response to iron deficiency (-Fe) is indicated as increased (+) or decreased (-). Transmembrane domains were predicted using the program TMPred (Hofmann and Stoffel, 1993). The gene number in *Chlamydomonas* was provided if known.

	-Fe	kDa	TM domains	Peptide sequences	Protein identity
1	+	150	1-2	DVLLEYTDASFTTVKPR (DL/VE/NN)VSLHPHGVR LQWNYAPSGR	multicopper ferroxidase FLP (FOX1) (Herbik *et al*., 2002b)
2	+	100	9-12	IGVTVTAGTNAATGAASK ALAAPPPAHVPTAAAYISTGRPDLK VTFVVSVSR NSGGVAASLSR LASLTPAFSR SASSGAAAAAASQR	ferric reductase-like (*C. reinhardtii*) (C_40004)
3	-	85	1?	AIYYITGESR	heat shock protein HSP90 (*C. reinhardtii*) (C_730014)
4	+	80	2-3	EAYLQAR GLFGPLTSR SPTLNDLRSIVDAAAQSLLPYAR SYIDAQVAAGTLVGAVR	unknown function (C_790051)
5	+	68	?	peptide fragments	heat shock protein
6	+	59	1?	AIDYLNTLDR ATTEPEIWNAIKFGTVLENVDYNPVTR ASYPIEFMNNAR ILTGGPQPIAK	PEP carboxykinase (*C. reinhardtii*) (C_130064)
7	-	56	none	VTGVNVFPTTRQISSSSVQFLQDR YVATFFNR NSAVSPLVAWIYYQPK QYVQDAGGR ALYGLTDAAALTDLKFLANQR	FAP24 (found in flagellar proteome) unknown function (*C. reinhardtii*) (C_50005)
8	+	21	2	AAIDQNSWSFMLNR SMDEFVAYLK	unknown function (C_2700003)

3.3 RT-PCR analysis of gene expression in iron-deficient *Chlamydomonas*

We have investigated the transcript abundance of 4 polypeptides that were detected by SDS-PAGE as being increased under Fe deficiency (Figure 19-5). Two of these polypeptides have known functions in Fe homeostasis [FLP (Fox1p) and ferritin (Fer1p)]. The other 2 polypeptides have unknown functions (80 kDa and 21 kDa proteins). In the *Chlamydomonas* genome, there is a second gene, *Fox2*, with 54% sequence identity to *Fox1*. The expression of *Fox2* was also included in this analysis. For *Fox1* an increased transcript was evident already after 30 min of Fe-deficient growth, and the signal was maximal between 12 and 24 h. Interestingly, after 1 h following re-supply of Fe, the transcript decreased to the Fe sufficient level but then increased again up to 24 h, the last time-point taken. This behavior following re-supply of Fe was also observed for the transcript to the 80 and 21 kDa proteins. Expression of the second putative ferroxidase (*Fox2*) was less sensitive to Fe supply. Expression increased transiently following 30 min of Fe starvation but decreased again to near control levels thereafter, only to increase again between 24 and 48 h of Fe deficiency (Figure 19-5). In general, expression of *Fox2* was only increased after prolonged Fe starvation; a fact that might support a function for *Fox2* at later events in Fe homeostasis. Although the 21 kDa polypeptide was clearly increased in electrophoretic analyses (Figure 19-4), the expression of the gene encoding this polypeptide was not greatly altered (Figure 19-5).

Also of interest was the behaviour of the 80 kDa protein, whose expression profile resembled that of *Fox1* (Figure 19-5). The 80 kDa transcript was expressed weakly under Fe sufficient conditions, increased after withdrawal of Fe and reached an expression maximum between 24 and 48 h. Although the function of this polypeptide is unknown, based on its similar behavior to Fox1p (FLP), it may be directly involved in Fe homeostasis. Finally, the expression of the ferritin gene, *Fer1*, was also clearly increased under Fe deficiency. This observation confirmed the previous observations of La Fontaine *et al.* (2002). Thus, for the polypeptides investigated, including the putative reductase (not shown) and with the exception of the 21 kDa polypeptide, observations at the proteome could be confirmed by RT-PCR analysis.

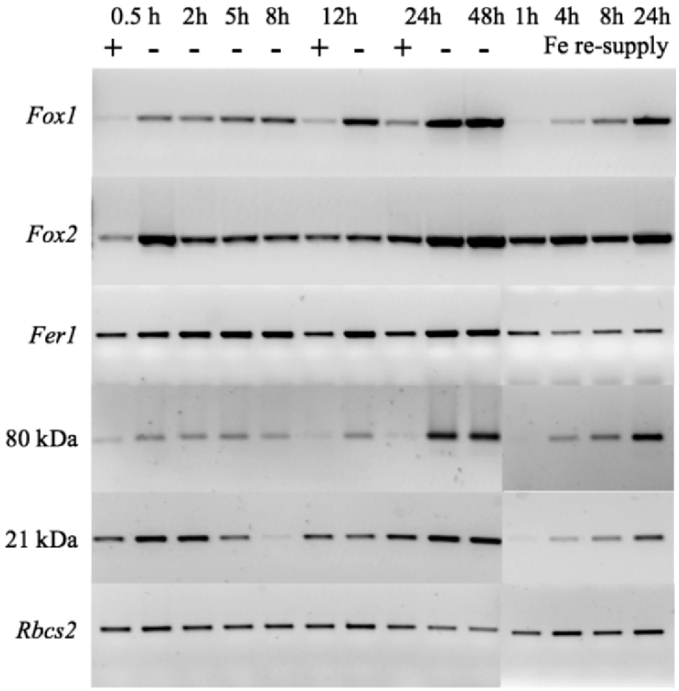

Figure 19-5. RT-PCR analysis of selected genes whose corresponding polypeptides were increased in the electrophoretic analysis. Total RNA from *Chlamydomonas* was isolated as described and reverse transcribed. Specific sequences were amplified by standard PCR procedures using gene-specific primers. Rbcs2 served as the loading control and its expression was not altered under iron starvation.

4. DISCUSSION

The mechanism of response of *Chlamydomonas* to Fe deficiency resembles that found in yeast. Fe^{3+} is initially reduced by a Fe^{3+}-chelate reductase and a ferroxidase/permease catalyzes uptake. With regard to transport, the ferroxidase-permease mechanism in yeast and presumably in *Chlamydomonas* differs significantly from the high-affinity Fe^{2+} transport in higher plants, which is catalyzed by IRT1p. At present there is no indication that a ferroxidase directly participates in high-affinity Fe uptake in plants. However, Hoopes and Dean (2004) reported the presence of a laccase-like ferroxidase that could potentially minimize the levels of Fe^{2+} and thereby the production of reactive oxygen species in the cells.

4.1 Proteome adaptation to iron deficiency

Using a proteomics approach, several polypeptides were identified whose synthesis was regulated by Fe deficiency. However, this approach alone has not provided a clear understanding of the mechanism of adaptation to Fe starvation. Important classes of regulatory proteins such as transcription factors and signaling proteins were not visualized on 2-dimensional gels, and the separation and resolution of hydrophobic membrane proteins was not adequate to allow routine analysis. In spite of the fact that membrane proteins have been detected using a 2-dimensional analysis, proteins that were clearly synthesized under Fe deficiency (e.g. Fe^{3+}-chelate reductase and Fe permease) were not detected (Reinhardt and Buckhout, unpublished).

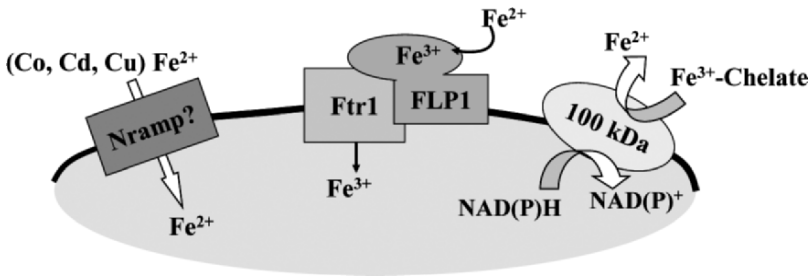

Figure 19-6. Model for the mechanism of high-affinity iron uptake in *Chlamydomonas.*

Apart from changes in the proteome that could be clearly assigned to regulation of Fe homeostasis, the majority of the polypeptide alterations could be ascribed to protein degradation and potentially to adaptation of the cells to dissimilation. Direct measurement of chlorophyll content in Fe deficient cells grown under identical conditions as used in this study showed that after 24 h Fe-deficient growth, chlorophyll was reduced by approximately 35% (Reinhardt and Buckhout, unpublished). Thus, the appearance of OEE subunits, the increase in ferritin and a heat shock protein and the decrease in translation/initiation factors are all consistent with protein breakdown and decreased protein synthesis. PEP carboxykinase and isocitrate lyase were both increased under Fe deficiency. These increases may indicate a redirection of C from lipid stores to glucose synthesis; an alteration in primary metabolism that may have resulted in decreased photosynthetic C fixation.

4.2 Ferroxidases in iron transport

Ferroxidases are members of a family of multicopper oxidases that oxidizes Fe^{2+} and catalyzes the reaction: 4 Fe^{2+} + O_2 + 4 H^+ –> 4 Fe^{3+} + 2 H_2O. In general, the catalytic portion of multicopper oxidases contains 4 Cu atoms that are classified into three types based on spectroscopic properties. Type 1 is the blue Cu site that is characterized by an intense absorption at approximately 600 nm (Quintanar *et al.*, 2004). The crystal structures of ceruloplasmin and laccase reveal that the type 1, blue copper site is separated from a 3 Cu cluster composed of 1 type 2 and 2 type 3 Cu atoms by ca. 13 Å (Blackburn *et al.*, 2000). The type 1 Cu^{2+} site is presumably the site for oxidation of Fe^{2+}, while O_2 reduction takes place at the 3 Cu cluster (Blackburn *et al.*, 2000).

The high-affinity Fe transport system of *Saccharomyces* and other yeast species is specific for Fe, is transcriptionally regulated and requires primarily 2 proteins, Ftr1p and Fet3p. (Askwith *et al.*, 1994). Expression of both Ftr1p and Fet3p is necessary for high affinity Fe transport (Stearman *et al.*, 1996). Fet3p is a copper-requiring enzyme with a membrane signal sequence and one transmembrane domain at the carboxyl terminus (de Silva *et al.*, 1995) Both Fet3p and Ftr1p are dependent upon one another for maturation. When Fet3p is absent, Ftr1p fails to localize to the plasma membrane, and without Ftr1p, Fet3p is not loaded with copper and is not active (Stearman *et al.*, 1996). Although the Fe permease in *Chlamydomonas* has not been characterized in such detail, there appears to be a Ftr1 homologue, which was induced under Fe deficiency (La Fontaine *et al.*, 2002; Reinhardt and Buckhout, unpublished).

In *Saccharomyces* a second ferroxidase, FET5p, which presumably is involved in Fe transport across the vacuolar membrane, has been identified (Urbanowski and Piper 1999). In addition to FET5p, an Fe transporter homologue, Fth1p, which shows sequence similarity to Ftr1p on the plasma membrane, was also localized on the vacuolar membrane. As with Ftr1p/Fet3p complex, Fth1p/Fet5p co-precipitate and disruption of the *FET5* gene results in the accumulation of Fth1p in the endoplasmic reticulum (Urbanowski and Piper 1999). Since Fet5p is oriented with the oxidase domain located extracytoplasmically, the Fth1p/Fet5p complex likely mobilizes Fe from the vacuole.

In *Chlamydomonas*, FLP (Fox1p) functions in an analogous manner to Fet3p in *Saccharomyces*. It has a putative transmembrane domain near the N terminus of the protein with a possible second transmembrane domain near the C terminus (TMPred). In addition to *Fox1*, a second putative ferroxidase, *Fox2*, has been identified by searching the *Chlamydomonas* genome. Fox1 and Fox2 have 54% sequence identity. Fox2 lacks an approximately 100

amino acid region at the N terminus of Fox1 and is induced under Fe-deficient growth, although most significantly after prolonged deficiency. Thus a role for Fox2 in Fe homeostasis seems likely but has as yet not been determined.

As stated, the mechanism of Fe uptake in *Chlamydomonas* resembles in many respects that found in yeast. We have incorporated the available information for Fe transport at the plasma membrane into a model shown in Figure 19-6. We have tentatively included the 100 kDa protein identified in this study as the Fe^{3+}-chelate reductase and the product of the gene *Ftr1* (La Fontaine *et al.*, 2002) as the Fe^{3+} permease. FLP (Fox1p) is most likely the ferroxidase involved in transport at the plasma membrane, since *Fox2* is expressed at much lower levels than *Fox1* and is less responsive to Fe supply (Reinhardt and Buckhout, unpublished). A low-affinity Fe transport system is clearly present in *Chlamydomonas* (Herbik *et al.*, 2002b); however, little is known as to the permeases that catalyze this reaction. In our model we have tentatively assigned this role to Nramp homologues based on the work of Rosakis and Köster (2004). Attempts to confirm these hypotheses are in progress.

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Chapter 20

MOLECULAR ANALYSIS OF IRON-DEFICIENT GRAMINACEOUS PLANTS

TAKANORI KOBAYASHI[1,2], NAOKO K. NISHIZAWA[1,2] and SATOSHI MORI[1]

[1]Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan. E-mail: mori@niad.ac.jp; [2]Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Corporation

Abstract: Graminaceous plants use mugineic acid family phytosiderophores (MAs), which are natural Fe(III) chelators, to acquire sparingly soluble Fe from the rhizosphere. The response of graminaceous plants to Fe-deficient conditions includes various processes involved in Fe homeostasis, the molecular components of which have been elucidated in recent years. The main processes are: (1) biosynthesis of MAs, (2) secretion of MAs, (3) uptake of Fe from the rhizosphere, (4) translocation and storage of Fe, and (5) regulation and signaling of the process. Regulation and signaling links the overall response to Fe availability and is thus of great importance. We recently identified two novel, Fe-deficiency-responsive, *cis*-acting elements, IDE1 and IDE2, aiding in the elucidation of the underlying molecular mechanisms. The future production of Fe-deficiency-tolerant or high Fe-containing crops will be largely dependent on a comprehensive understanding of the molecular components of Fe homeostasis in graminaceous plants.

Key words: iron deficiency-inducible expression; iron-deficiency-responsive elements; mugineic acid family phytosiderophores; roots; transporter

1. INTRODUCTION

Since the discovery by Takagi (1976) of the Fe(III)-solubilizing capacity of root washings from Fe-deficient rice and oat plants, the mugineic acid family phytosiderophores (MAs) have been identified as natural Fe(III) chelators synthesized in the roots of graminaceous plants. Römheld and Marschner (1986) termed the Fe-acquisition mechanism that is specific to

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graminaceous plants 'Strategy II'; the molecular components of this mechanism have subsequently been elucidated in recent years. In this chapter, we first summarize the molecular components and related genes that have been identified or inferred through physiological and molecular studies on Fe-deficient graminaceous plants. As the components are regulated in response to Fe availability, our recent identification of the novel, Fe-deficiency-responsive, *cis*-acting elements IDE1 and IDE2 represents a milestone in furthering the understanding of the mechanism at the molecular level. Therefore, we next review the process of identifying these elements. Finally, future perspectives on the clarification of the overall mechanism of Fe homeostasis in graminaceous plants and the production of Fe-deficiency-tolerant or high Fe-containing crops are discussed.

2. MOLECULAR COMPONENTS OF THE IRON-ACQUISITION MECHANISM IN GRAMINACEOUS PLANTS

2.1 Genes involved in the biosynthesis of MAs

2.1.1 Biosynthetic pathway of MAs

The synthesis and secretion of MAs are specific to graminaceous plants and are strongly enhanced by Fe deficiency. The identification of the molecular components involved in the biosynthesis of MAs has been realized on the basis of extensive physiological studies that have focused on the identification of MAs and their biosynthetic pathway. To date, nine kinds of MAs have been identified (Nomoto *et al.*, 1987; Ma *et al.*, 1999; Ueno *et al.*, 2004) and their biosynthetic pathways have been investigated both *in vivo* (Mori and Nishizawa, 1987, 1989; Kawai *et al.*, 1988; Mori *et al.*, 1990; Ma and Nomoto, 1993; Ma *et al.*, 1999) and *in vitro* (Shojima *et al.*, 1989, 1990).

Methionine is the primary precursor of MAs (Mori and Nishizawa, 1987). The methionine cycle has been shown to work vigorously in roots to meet the increased demand for methionine in the synthesis of MAs (Ma *et al.*, 1995). The methionine cycle and the biosynthetic pathway of MAs are shown in Figure 20-1. By monitoring real-time ^{11}C-methionine movement using a positron-emitting tracer imaging system (PETIS), methionine as a precursor of MAs was found to originate in the roots but not in the shoots of barley plants (Nakanishi *et al.*, 1999; Bughio *et al.*, 2001).

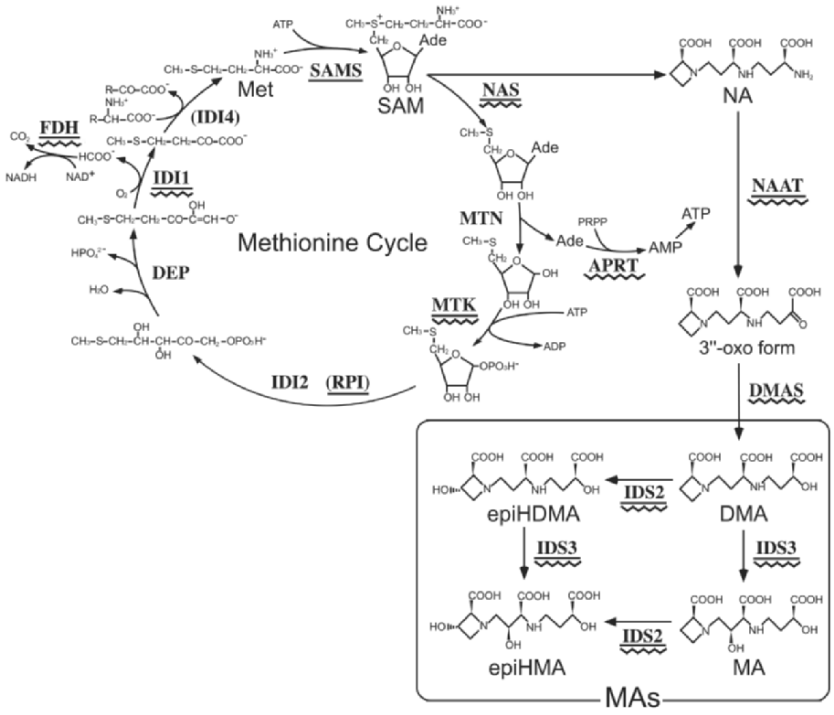

Figure 20-1. The biosynthetic pathway of MAs and the methionine cycle in graminaceous plants (original figure: Kobayashi *et al.*, 2005). The expression of the depicted enzymes is induced by Fe deficiency in rice and/or barley. The promoters of the rice and/or barley genes corresponding to the underlined enzymes contain IDE1-like (straight line) or IDE2-like (wavy line) sequences (see section 3). Met, methionine; Ade, adenine; SAM, S-adenosyl-Met; SAMS, S-adenosyl-Met synthetase; NAS, nicotianamine synthase; NAAT, nicotianamine aminotransferase; DMAS, 2'-deoxymugineic acid synthase; IDS2 and IDS3, dioxygenases catalyzing the hydroxylation of MAs; MTN, methylthioadenosine/S-adenosyl homocysteine nucleosidase; MTK, methylthioribose kinase; IDI2, eukaryotic initiation factor 2B-like methylthioribose-1-phosphate isomerase; RPI, putative ribose-5-phosphate isomerase; DEP, methylthioribulose-1-phosphate dehydratase-enolase-phosphatase; IDI1, 2-keto-methylthio-butyric-acid-forming enzyme; IDI4, putative aminotransferase catalyzing the synthesis of Met from 2-keto-methylthiobutyric acid; FDH, formate dehydrogenase; APRT, adenine phosphoribosyltransferase. To date, five other kinds of MAs have been identified.

2.1.2 Nicotianamine synthase (NAS) genes

Nicotianamine synthase (NAS) is a unique enzyme that catalyzes the trimerization of three *S*-adenosyl-L-methionine (SAM) molecules, with the release of adenine and the formation of an azetidine ring, to produce nicotianamine (NA), which is a common metal chelator used by all plant species (Noma and Noguchi, 1976; von Wirén *et al.*, 1999). *NAS* genes were

first isolated in barley (*HvNAS1-7*) through the establishment of an NAS activity assay (Higuchi *et al.*, 1994) and enzyme purification from Fe-deficient barley roots (Higuchi *et al.*, 1999). Since then, *NAS* genes have again been isolated from barley (*nashor1, 2*; Herbik *et al.*, 1999), as well as from rice (*OsNAS1-3*; Higuchi *et al.*, 2001a), maize (*ZmNAS1-3*; Mizuno *et al.*, 2003), and the non-graminaceous species tomato (*CLN*; Ling *et al.*, 1999) and *Arabidopsis* (*AtNAS1-3*; Suzuki *et al.*, 1999; *AtNAS4*; Wintz *et al.*, 2003), in which NA is proposed to be involved in the internal transport of metal ions (Scholz *et al.*, 1992; Stephan and Scholz, 1993; Pich *et al.*, 1994; Stephan *et al.*, 1994; Ling *et al.*, 1999; Takahashi *et al.*, 2003). Graminaceous *NAS* genes are categorized into two groups based on their expression patterns and positions on an unrooted phylogenic tree (Figure 20-2; Mizuno *et al.*, 2003). The first group of *NAS* genes, which includes *HvNAS1*, *ZmNAS1*, *ZmNAS2*, *OsNAS1,* and *OsNAS2,* is strongly induced by Fe deficiency in the roots and is therefore expected to be involved in MAs biosynthesis for the acquisition of Fe from the rhizosphere. Among these genes, *OsNAS1* and *OsNAS2* are also induced in Fe-deficient leaves, while *HvNAS1*, *ZmNAS1*, and *ZmNAS2* are expressed predominantly in the roots (Higuchi *et al.*, 1999, 2001a; Inoue *et al.*, 2003; Mizuno *et al.*, 2003). On the other hand, the second group of *NAS* genes, including *ZmNAS3* and *OsNAS3*, is not strongly induced in Fe-deficient roots and is thought to be involved in Fe translocation within the plant (Inoue *et al.*, 2003; Mizuno *et al.*, 2003).

To clarify the tissue-specific localization of *NAS* expression, *OsNAS1*, *OsNAS2*, and *OsNAS3* promoters were individually fused to the β-glucuronidase gene (*GUS*) and introduced into rice (Inoue *et al.*, 2003). Typical results of histochemical analyses of these transgenic rice plants are shown in Figure 20-3. The expression of *OsNAS1* and *OsNAS2* was observed in companion cells and pericycle cells adjacent to the protoxylem of Fe-sufficient roots (Figure 20-3A, B). *OsNAS1* and *OsNAS2* expression was strongly induced by Fe deficiency in all cells of Fe-deficient roots (Figure 20-3C, D) and severely chlorotic leaves (Figure 20-3F), as well as in the vascular bundles of green leaves (Figure 20-3E). On the other hand, *OsNAS3* expression was restricted to the pericycle and companion cells in the roots and companion cells in the leaves irrespective of Fe status. These histochemical observations strongly suggest that NAS and NA play a role in the long-distance transport of Fe within rice plants, in addition to their role in the biosynthesis of MAs (Inoue *et al.*, 2003).

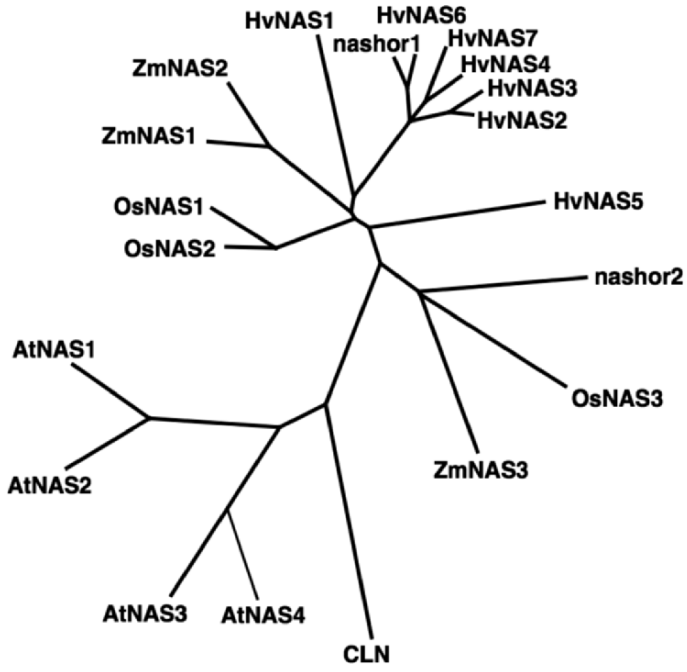

Figure 20-2. Unrooted phylogenic tree for NAS amino acid sequences (original figure: Mizuno *et al.*, 2003).

2.1.3 Nicotianamine aminotransferase (NAAT) genes

Nicotianamine aminotransferase (NAAT) catalyzes the transamination of NA to produce the 3''-oxo intermediate and is a key enzyme in the biosynthetic pathway of MAs, as this is the first step specific to graminaceous plants. The isolation of *NAAT* genes from barley roots was achieved using a strategy similar to that used to clone *NAS*, i.e. the establishment of an enzyme activity assay (Ohata *et al.*, 1993) and enzyme purification (Kanazawa *et al.*, 1995; Takahashi *et al.*, 1999). Takahashi *et al.* (1999) isolated two barley *NAAT* genes, *HvNAAT-A* and *HvNAAT-B*, which are tandemly located on the barley genome. The expressions of both *HvNAAT-A* and *HvNAAT-B* are strongly induced by Fe deficiency and occur almost exclusively in the roots, similar to *HvNAS1* expression.

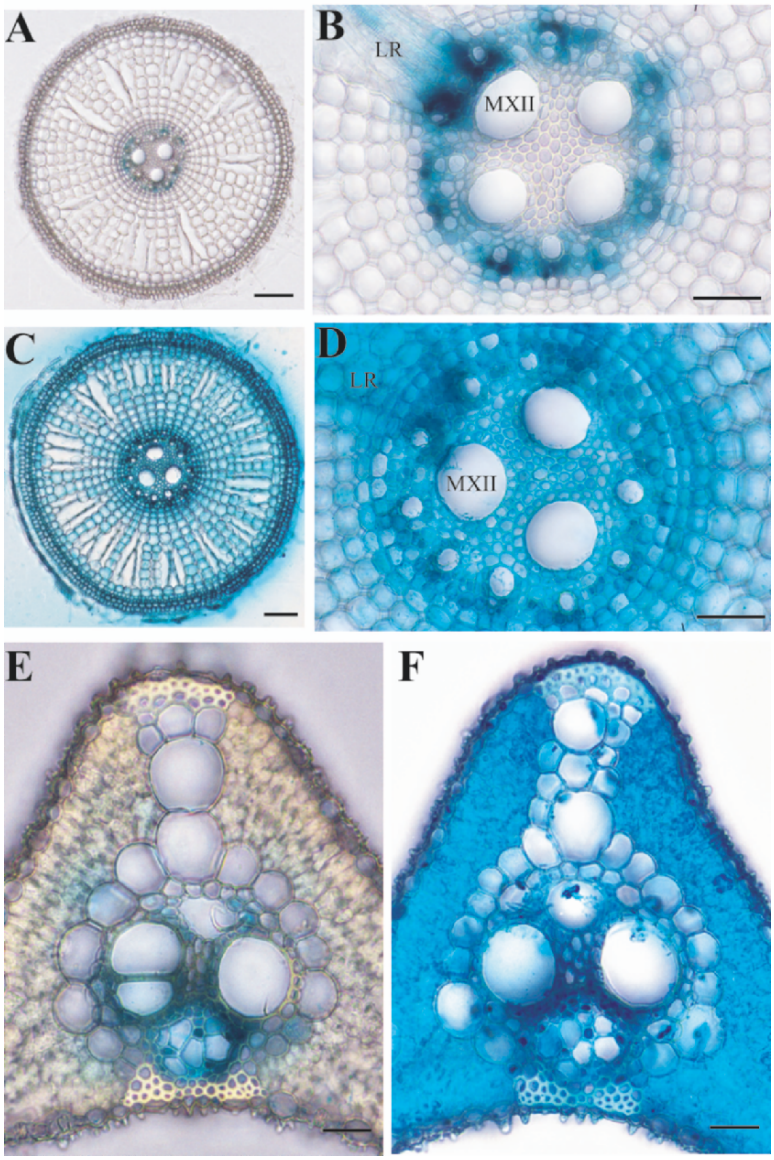

Figure 20-3. Histochemical localization of *OsNAS1* promoter-*GUS* expression in transgenic rice plants (original figure: Inoue *et al.*, 2003): **(A)** Transverse section of Fe-sufficient roots; **(B)** Enlarged part of Fe-sufficient root stele; **(C)** Transverse section of Fe-deficient roots; **(D)** Enlarged part of Fe-deficient root stele; **(E)** Green leaf of Fe-deficient plants, and **(F)** Chlorotic leaf of Fe-deficient plants. LR, lateral root; MX II, metaxylem II. Scale bars = 100 µm for **(A)** and **(C)**; 50 µm for **(B)**, **(D)**, **(E)** and **(F)**.

Rice possesses five putative *NAAT* genes (*OsNAAT1-5*), among which only *OsNAAT1* is induced by Fe deficiency in roots and shoots (Inoue *et al.*, 2004). Transgenic rice plants harboring the *OsNAAT1* promoter-*GUS* fusion were analyzed to localize expression. The expression patterns in roots and leaves were very similar to those of *OsNAS1* and *OsNAS2*, suggesting the possible importance of MAs, in addition to NA, in the long-distance transport of Fe in rice plants (Inoue *et al.*, 2004). Moreover, the expression of *OsNAAT1* as well as *OsNAS* was also observed in the reproductive organs, including flowers and maturing seeds, suggesting the importance of NA and MAs in embryogenesis and metal translocation in these organs (Takahashi *et al.*, 2004).

2.1.4 Genes encoding the dioxygenases for the hydroxylation of MAs

In contrast to the establishment of an *in vitro* system for characterizing the conversion of methionine to 2'-deoxymugineic acid (DMA) (Shojima *et al.*, 1989, 1990), the steps for the conversion of DMA to other MAs have not been successfully reproduced *in vitro*; instead, these steps have been proposed from genotypic variances in the capacity for MAs biosynthesis among graminaceous plants (Figure 20-1; Mori and Nishizawa, 1987, 1989; Mori *et al.*, 1990; Ma and Nomoto, 1993; Ma *et al.*, 1999). All species of graminaceous plants investigated to date share the capacity to produce and secrete DMA; DMA is then converted to other MAs in a manner dependent on the species. Barley secretes mugineic acid (MA), 3-epihydroxy-2'-deoxymugineic acid (epiHDMA), and 3-epihydroxymugineic acid (epiHMA) in addition to DMA. Rye secretes DMA, MA, and 3-hydroxymugineic acid (HMA), while oat secretes DMA and avenic acid A. Rice, wheat, maize, and sorghum usually secrete only DMA, although some kinds of ancestral wheat also secrete unknown MAs (Singh *et al.*, 2000), and 'old plants' of rice (cv. Honenwase grown under Fe-sufficient conditions for two months and then under Fe-deficient conditions for one month) secrete MA and epiHMA in addition to DMA (Mori *et al.*, 1991).

The chromosomal locations of the genes coding these hydroxylation enzymes were examined using wheat (cv. Chinese Spring)-barley (cv. Betzes) addition lines. The gene for hydroxylation at the C-2' position of MAs, i.e., conversion from DMA to MA and from epiHDMA to epiHMA, is located on the long arm of barley chromosome 4H. The gene for hydroxylation at the C-3 position (DMA to epiHDMA and MA to epiHMA) is located on the long arm of barley chromosome 7H (Mori and Nishizawa, 1989; Ma *et al.*, 1999). Similarly, using wheat (cv. Chinese Spring)-rye (cv. Imperial) addition lines (Mori *et al.*, 1990), rye chromosome 5R was

identified as carrying the genes for the conversion from DMA to MA and from MA to HMA.

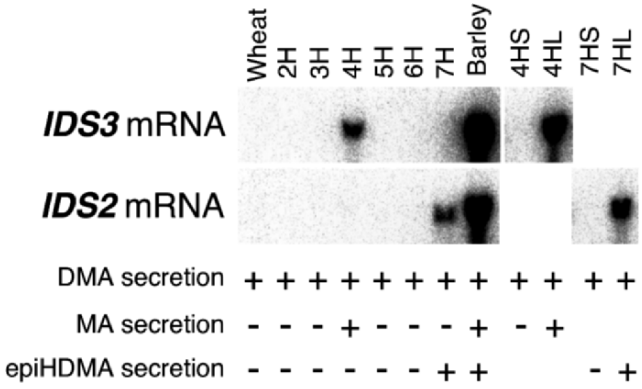

Figure 20-4. The expression of *IDS3* and *IDS2* in the Fe-deficient roots of wheat (cv. Chinese Spring)-barley (cv. Betzes) addition lines in comparison with the secretion capacity of MA and epiHDMA (original figure: Nakanishi *et al.*, 2000). Wheat (cv. Chinese Spring)-barley (cv. Betzes) ditelosomic addition lines for 2H, 3H, 4H, 4HS, 4HL, 5H, 6H, 7H, 7HS, and 7HL were grown under conditions of Fe deficiency and were then subjected to Northern blot analysis using *IDS3* or *IDS2* cDNA as a probe.

Using the differential hybridization method with mRNA from Fe-deficient and Fe-sufficient barley roots, we cloned *IDS* (iron-deficiency specific) genes specifically expressed in Fe-deficient barley roots (Okumura *et al.*, 1991, 1994; Nakanishi *et al.*, 1993). Among these, *IDS2* and *IDS3* are homologous to 2-oxoglutarate-dependent dioxygenases, suggesting their possible involvement in the hydroxylation of MAs. Moreover, Northern and Western analyses showed that *IDS3* was expressed in Fe-deficient roots of barley and rye, as well as in Add-4H and 4HL wheat (cv. Chinese Spring)-barley (cv. Betzes) addition lines, all of which secrete MA (Mori and Nishizawa, 1989; Mori *et al.*, 1990; Ma *et al.*, 1999). *IDS3* was not expressed in other plants (rice, wheat, maize, oat, and sorghum) that secrete DMA but not MA (Figure 20-4; Nakanishi *et al.*, 2000). Similarly, *IDS2* was specifically expressed in Fe-deficient roots of barley and Add-7H and 7HL wheat (cv. Chinese Spring)-barley (cv. Betzes) addition lines (Figure 20-4; Nakanishi *et al.*, 2000), which possess the capacity to produce epiHDMA from DMA (Ma *et al.*, 1999). These results strongly suggest that IDS3 is the enzyme that hydroxylates the C-2' positions of DMA and epiHDMA, while IDS2 hydroxylates the C-3 positions of DMA and MA (Figure 20-1; Nakanishi *et al.*, 2000). As attempts to detect the enzyme activity of IDS3 *in vitro* were unsuccessful, we chose to determine the function of IDS3 *in vivo* using transgenic rice plants. Transgenic rice plants introduced with

IDS3 cDNA (cv. Nipponbare) or a barley genomic fragment containing *IDS3* (cv. Tsukinohikari) secreted MA in addition to DMA under Fe-deficient conditions, while non-transformants secreted only DMA. This showed that IDS3 is the "MA synthase" that hydroxylates DMA to produce MA (Kobayashi *et al.*, 2001). The hydroxylation of MAs in barley may contribute to barley's high tolerance for Fe deficiency, possibly by stabilization of the Fe(III)-MAs complexes, as suggested by von Wirén *et al.* (2000), and possibly by promoting DMA synthesis from its precursor (Kobayashi *et al.*, 2001).

The expressions of *IDS3* and *IDS2* are strongly induced in barley roots with the progression of Fe deficiency (Nakanishi *et al.*, 1993, 2000; Okumura *et al.*, 1994). *IDS2* expression is also induced in barley roots under conditions of Mn or Zn deficiency (Okumura *et al.*, 1994). *In situ* hybridization analysis indicated that *IDS2* mRNA was strongly expressed in the endodermis, pericycle, and cortex of Fe-deficient adventitious barley roots (Yoshihara *et al.*, 2003).

2.1.5 *S*-adenosyl-L-methionine synthetase (*SAMS*) genes

Barley *S*-adenosyl-L-methionine synthetase (*SAMS*) genes were isolated using the consensus region of previously identified *SAMS* genes. Neither *SAMS* expression nor SAMS enzyme activity was found to be induced by a two-week treatment of Fe deficiency in barley roots (Takizawa *et al.*, 1996). However, recent microarray and Northern blot analyses revealed that barley and rice *SAMS* genes are induced in Fe-deficient roots (Negishi *et al.*, 2002; Kobayashi *et al.*, 2005).

2.1.6 Genes encoding the reductase involved in DMA synthesis

Until recently, the reduction step from the 3"-oxo intermediate to DMA was not fully characterized. We have attempted to clone the corresponding genes, DMA synthase (*DMAS*), by PCR amplification using conserved sequences of the aldo-keto reductase superfamily. We have also identified putative *DMAS* genes in a microarray analysis as genes that are upregulated in Fe-deficient rice roots (unpublished). Demonstration of enzyme activity of the protein products in synthesizing DMA from the 3"-oxo form, and characterization of expression profiles of the *DMAS* genes is currently in progress.

2.1.7 Formate dehydrogenase (FDH) and adenine phosphopribosyltransferase (APRT) genes

We compared proteins of Fe-sufficient and Fe-deficient barley roots using 2D-PAGE. Peptide sequencing of the induced proteins revealed that formate dehydrogenase (FDH) and adenine phosphoribosyltransferase (APRT), as well as the IDS3 protein, were induced in Fe-deficient roots (Suzuki *et al.*, 1998). Both FDH and APRT are thought to function in scavenging the by-products (formate and adenine) that are released during the methionine cycle (Mori, 1999; Figure 20-1), thus supporting the production of MAs. The enzyme activities of both FDH and APRT were shown to be induced in Fe-deficient roots (Suzuki *et al.*, 1998; Itai *et al.*, 2000). The barley cDNAs encoding these enzymes were cloned, and their expression was confirmed to occur almost exclusively in the roots and to be induced by Fe deficiency (Suzuki *et al.*, 1998; Itai *et al.*, 2000). The expression of the *FDH* gene, as well as the Fe-deficiency-inducible alcohol dehydrogenase (*Adh*) gene, is also induced for energy production under anaerobic stress, which reflects the physiological anemia in Fe-deficient roots owing to the inability to synthesize the porphyllin ring as a precursor of heme (Suzuki *et al.*, 1998; Mori, 1999). Recently, rice putative homologs of the *FDH* and *APRT* genes were identified, and their expression was shown to be induced by Fe deficiency both in roots and leaves (Kobayashi *et al*., 2005; Suzuki *et al*., unpublished).

2.1.8 Genes encoding enzymes in the methionine cycle

Using a revised differential hybridization screening, iron-deficiency induced (*IDI*) genes were cloned from barley roots (Yamaguchi *et al*., 2000a, 2000b, 2002). Among these, the *IDI1* gene encodes the enzyme that catalyzes the formation of 2-keto-methylthiobutyric acid in the methionine cycle (Figure 20-1; Yamaguchi *et al.*, 2000a). Recent *in silico* analysis suggested that the *IDI2* gene, and putatively the *IDI4* gene as well, also encode the enzymes of the methionine cycle (Kobayashi *et al*., 2005; Suzuki *et al*., unpublished). Similar to the *FDH* and *APRT* genes, the *IDI1* and *IDI2* genes are expressed almost exclusively in the roots, whereas the expression of their rice homologs are induced by Fe deficiency both in roots and leaves (Kobayashi *et al*., 2005; Suzuki *et al*., unpublished).

Recently, microarray analysis has allowed us to investigate the Fe-deficiency-regulated expression of other genes putatively encoding enzymes in the methionine cycle, which were deduced by their homology to bacterial genes of the methionine salvage pathway. We found that the expression of rice genes involved in all of the predicted steps of the methionine cycle is

entirely induced by Fe deficiency in the roots. The expression of most of these genes was found to be induced by Fe deficiency also in the leaves (Kobayashi *et al.*, 2005).

2.2 Genes involved in the secretion of MAs

2.2.1 Particular vesicles in iron-deficient root cells as the sites of MAs synthesis

In contrast to the biosynthetic pathway of MAs, the molecular components involved in the secretion of MAs remain unclear. We have shown that MAs are secreted in the form of monovalent anions via anion channels using the potassium gradient (Sakaguchi *et al.*, 1999). Takagi *et al.* (1984) reported that the secretion of MAs in barley follows a distinct diurnal rhythm. A peak in secretion occurs just after initial illumination and ceases within 2-3 h. In parallel with this diurnal secretion, certain vesicles in the root cells of Fe-deficient barley change in shape (Nishizawa and Mori, 1987). The vesicles remain swollen until the onset of the secretion of MAs and become shrunken by the end of secretion. These vesicles are thought to be derived from the rough endoplasmic reticulum and are proposed to be the site of MAs synthesis. This hypothesis was further supported when the subcellular localizations of the enzymes participating in the MAs biosynthesis were investigated using a transient expression system with green fluorescent protein (GFP) fusion and immunocytochemistry (Itai *et al.*, 2000; Kobayashi *et al.*, 2000; Nishizawa *et al.*, 2000; Mizuno *et al.*, 2003). HvNAS1, ZmNAS1, ZmNAS2, HvNAAT-A, and HvIDS3 proteins fused to GFP were targeted to small vesicles, whereas HvAPRT1-GFP and ZmNAS3-GFP fusion proteins were detected in the cytoplasm. Immunocytochemical observations of Fe-deficient barley roots revealed that NAS was localized on the limiting membrane of the above-mentioned particular vesicles, and IDS3 was localized on the cytoplasmic side of the limiting membrane of the vesicles, while NAAT was present within the vesicles. It was supposed that SAM supplied from the cytoplasm was converted to DMA inside the vesicles and that DMA was pooled until sunrise, then hydroxylated in the vicinity of the vesicles, and secreted (Figure 20-5).

2.2.2 Possible molecular components involved in the secretion of MAs

To identify genes involved in the diurnal secretion of MAs in barley, a microarray analysis was performed; approximately 50 Fe-deficiency-inducible

genes showing diurnal changes in expression were identified (Negishi *et al.*, 2002). These included the *SAMS* and *NAS* genes, as well as genes possibly related to vesicle transport, including the ras-related GTP-binding protein *RIC1* and the ADP-ribosylation factor 1 (*ARF1*). Northern analysis showed that rice homologs of *RIC1* and *ARF1*, as well as the *OsNAS* and *OsNAAT* genes, also exhibit diurnal changes in expression (Nozoye *et al.*, 2004). These results suggest that the secretion of MAs in graminaceous roots is under the control of vesicular transport (Figure 20-5).

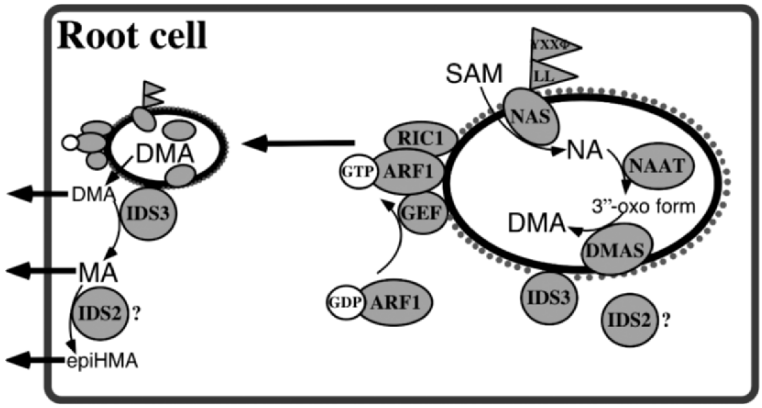

Figure 20-5. Model of MAs synthesis in particular vesicles and their vesicular transport for secretion (original figure: Nozoye *et al.*, 2004). We propose that MAs are synthesized in the rough endoplasmic reticulum-derived vesicles that appear in the cortex cells of Fe-deficient roots. MAs synthesized in these vesicles are localized to the cell boundaries facing the rhizosphere through a polar vesicle transport process involving ARF1 and RIC1 GTPase, leading to the diurnal secretion of MAs, possibly after hydroxylation. The LL and YXXΦ motifs conserved in the NAS proteins function in ARF GEF-mediated polar localization. The localization of DMAS and IDS2 proteins has not been experimentally investigated yet.

2.3 Genes involved in the transport of iron from the rhizosphere to the root cells

2.3.1 Genes encoding the iron(III)-MAs transporter

Early studies proposed the presence of a specific transport system for the Fe(III)-MAs complex in the plasma membrane of root cells in graminaceous plants (Römheld and Marschner, 1986; Mihashi and Mori, 1989). Efforts to isolate the corresponding transporter gene by functional complementation of yeast mutants were not successful. The maize *yellow stripe 1* (*ys1*) mutant is defective in Fe(III)-MAs uptake (von Wirén *et al.*, 1994); therefore, YS1 has been suggested to be the Fe(III)-MAs transporter. Molecular cloning of the

YS1 gene was performed by Curie *et al.* (2001) using a transposon-tagged population. A yeast strain defective in Fe uptake was complemented by the expression of *YS1* when supplied with Fe(III)-DMA. *YS1* expression in maize is increased in both roots and shoots under conditions of Fe deficiency, but is not strongly affected by Zn or Cu deficiency (Curie *et al.*, 2001; Roberts *et al.*, 2004). Schaff *et al.* (2004) investigated the precise transporting properties of YS1 in *Xenopus* oocytes by electrophysiological analysis. YS1 functions as a proton-coupled symporter for various DMA-bound metals including Fe(III), Zn(II), Cu(II), and Ni(II). YS1 also transports NA-chelated Ni(II), Fe(II), and Fe(III) complexes.

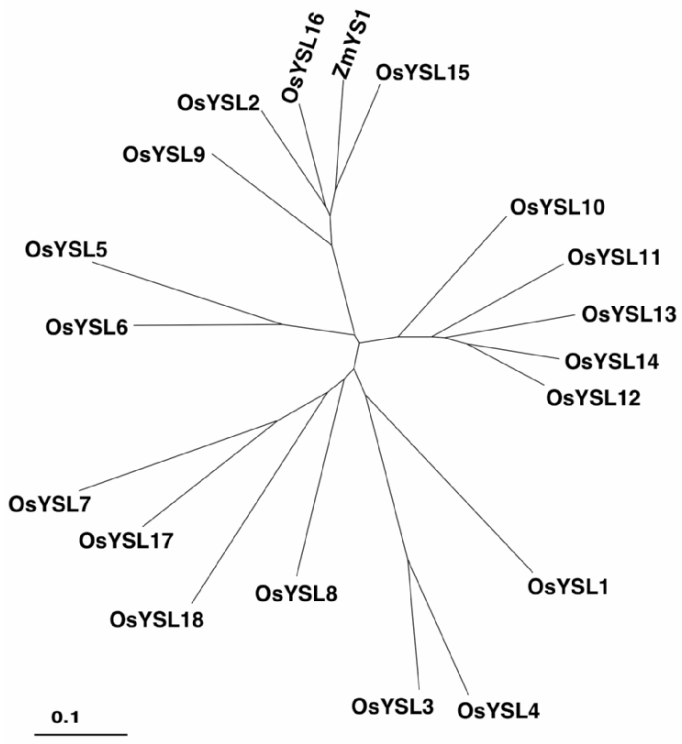

Figure 20-6. Unrooted phylogenic tree for ZmYS1 and 18 OsYSL amino acid sequences (original figure: Koike *et al.*, 2004).

Our search for *YS1* homologs in the rice genome database identified 18 putative *OsYSL* (*Oryza sativa YS1*-like) genes (Figure 20-6; Koike *et al.*, 2004). Northern analysis detected the transcripts of *OsYSL2*, *6*, *13*, *14*, *15,* and *16* in roots or leaves of Fe-deficient or Fe-sufficient rice plants. Among these, the expression of *OsYSL15* and *16* was induced in Fe-deficient roots, suggesting the possibility that these genes play a role in Fe-MAs uptake

from the rhizosphere. We are currently analyzing the transporting properties of these *OsYSL* genes.

2.3.2 Genes encoding the iron(II) transporter

In non-graminaceous plants, Fe transport from the rhizosphere to the root cell is mediated by an Fe(II) transporter. Eide *et al.* (1996) isolated the *Arabidopsis IRT1* gene, which is the dominant Fe(II) transporter in the Fe-uptake process. Various transporter genes homologous to *IRT1* have been found in all eukaryotic kingdoms (plants, animals, protists, and fungi) and were named zinc-regulated transporter, iron-regulated transporter-like protein (ZIP) family (Guerinot, 2000). Bughio *et al.* (2002) isolated a rice homolog of the *Arabidopsis IRT1* gene, *OsIRT1*, whose Fe(II)-transporting capacity was ascertained by functional complementation in yeast. The expression of *OsIRT1* is strongly induced in Fe-deficient rice roots. The OsIRT1-GFP fusion protein was localized in the plasma membrane of onion epidermal cells (Ishimaru *et al.*, 2004). Promoter-*GUS* analysis showed that *OsIRT1* was mainly expressed in the epidermis, endodermis, and inner layer of the cortex in Fe-deficient roots, as well as in companion cells of shoots. Moreover, the PETIS method revealed that rice is able to take up both Fe(III)-DMA and Fe(II). Thus, rice plants possess a system other than the MAs-based Strategy II for Fe uptake (Ishimaru *et al.*, 2004). Such a system to take up Fe(II) seems reasonable because rice is commonly grown under waterlogged conditions in which the dominant form of soil Fe is Fe(II) (Bughio *et al.*, 2002).

2.4 Genes involved in the translocation and storage of iron

2.4.1 Transporter genes involved in the transport of iron inside the plant body

The translocation of Fe and other minerals inside the plant body involves a sequence of processes that require various metal chelators and transporters (Clemens *et al.*, 2002; Hell and Stephan, 2003). The rice genome putatively contains 18 *OsYSL* genes, 13 *OsZIP* genes, and eight *OsNRAMP* genes (Gross *et al.*, 2003; Koike *et al.*, 2004). The natural resistance-associated macrophage protein (NRAMP) family transporters are found in bacteria, fungi, plants, and animals; some of them have been shown to function as transporters of various cations including Fe(II), Mn(II), Cd(II), and Zn(II) (Gunshin *et al.*, 1997; Chen *et al.*, 1999).

Among the 18 *OsYSL* genes, *OsYSL2* is expressed predominantly in Fe-deficient leaves (Koike *et al.*, 2004). The OsYSL2-GFP fusion protein was localized in the plasma membrane of onion epidermal cells (Figure 20-7A). Electrophysiological analysis in *Xenopus* oocytes showed that OsYSL2 transported Fe(II)-NA and Mn(II)-NA, but did not transport Fe(III)-MAs. An analysis using transgenic rice containing the *OsYSL2* promoter-*GUS* construct revealed that *OsYSL2* was expressed in root companion cells (Figure 20-7C, D) and phloem cells of the leaves and leaf sheaths (Figure 20-7E-H). Fe deficiency induced the expression of *OsYSL2* in all leaf tissues (Figure 20-7H). Strong expression of *OsYSL2* was also observed in the vascular bundles of flowers and in developing seeds (Figure 20-7I-N). These results suggest that OsYSL2 functions as an Fe(II)-NA transporter responsible for the phloem transport of Fe, including the translocation of Fe into the grain (Koike *et al.*, 2004). The characterization of the other *OsYSL* members is currently in progress.

The involvement of graminaceous *ZIP* and *NRAMP* genes in Fe translocation has not been well characterized. Ramesh *et al.* (2003) demonstrated that two of the OsZIP members, OsZIP1 and OsZIP3, possess a Zn(II)-transporting capacity. Graminaceous *ZIP* genes are expected to have diverse functions in the translocation of metals, including Fe(II) and Zn(II). Among the *Arabidopsis* and rice homologs of the *NRAMP* family genes, *AtNRAMP1*, *AtNRAMP3*, *AtNRAMP4,* and *OsNRAMP1* were reported to be able to complement yeast mutants defective in Fe uptake (Curie *et al.*, 2000; Thomine *et al.*, 2000). AtNRAMP3 was shown to be localized in the vacuolar membrane and was proposed to mobilize vacuolar metal pools to the cytosol (Thomine *et al.*, 2003). Similarly, graminaceous *NRAMP* genes are expected to be involved in subcellular compartmentalization of metals, including Fe and Mn.

Additionally, a function of homologs of the vertebrate IREG1 Fe exporter has been suggested in plant Fe homeostasis (Honsbein *et al.*, 2004). *IDI7*, which is an Fe-deficiency-responsive gene in barley roots that encodes a tonoplast-located ABC-type transporter, may also be involved in Fe translocation (Yamaguchi *et al.*, 2002).

2.4.2 Genes involved in the transport of iron: chelators

The chemical properties of Fe, including poor solubility and high reactivity, compel plants to use suitable chelating molecules inside the plant (Marschner, 1995; Clemens *et al.*, 2002). Physiological and molecular studies have indicated that one of the principal Fe chelators inside the plant is NA. As mentioned earlier, the expression of the *OsNAS* and *OsYSL2* genes in the vascular bundles, including companion cells (Figure 20-3, 7), strongly

suggests that NA plays a role in the long-distance transport of Fe (Inoue *et al.*, 2003; Koike *et al.*, 2004).

The necessity for NA in the transport of Fe, including phloem loading/unloading and the distribution of Fe from cells adjacent to the veins to the leaf lamina, has also been demonstrated in non-graminaceous plants through the observation of the tomato mutant *chloronerva*, which lacks NA as a result of a point mutation in the *NAS* gene (Becker *et al.*, 1995; Higuchi *et al.*, 1996; Ling *et al.*, 1999). Moreover, transgenic tobacco plants overexpressing the *HvNAAT* gene exhibited phenotypes similar to that of the *chloronerva* mutant, including severe interveinal chlorosis in young leaves, flower deformity, and sterility, owing to the exhaustion of NA (Takahashi *et al.*, 2003). The characterization of the transformants suggested the essential function of NA in intercellular and intracellular metal transfer in both vegetative and reproductive organs.

In addition to the central role of NA, MAs synthesized by graminaceous plants would also be involved in Fe translocation. The expression of *OsNAAT* genes in vascular bundles and maturing seeds supports this idea (Inoue *et al.*, 2004; Takahashi *et al.*, 2004). Large amounts of DMA are detected in rice phloem sap, where Fe may be chelated by DMA (Mori *et al.*, 1991). The characterization of the *YSL* family will aid in clarifying the role of MAs and NA in Fe translocation.

Organic acids are also common metal chelators inside the plant body. In particular, Fe(III)-citrate is believed to be the dominant form of Fe in the xylem sap (Tiffin, 1966; Brown and Chaney, 1971). Recently, an 11-kDa iron transport protein (ITP) was proposed as the Fe-transporting substance in the phloem sap of *Ricinus* (Krüger *et al.*, 2002), although it remains to be seen whether similar substances are present in graminaceous plants.

Recently, nitric oxide has been found to support Fe mobilization inside the plant, thus improving Fe nutritional status under Fe-limiting conditions (Graziano and Lamattina, 2005).

2.4.3 Relation to other micronutrients

As already mentioned, both Fe transporters and chelators often interact with a wide range of micronutrients other than Fe. Thus, Fe nutrition is closely linked to other micronutrients, which is often a matter of both interest and concern in agriculture. Among the Fe-deficiency-inducible genes that have been isolated from barley roots, the *IDS1* gene encodes metallothionein, which is a heavy metal-binding protein (Okumura *et al.*, 1991).

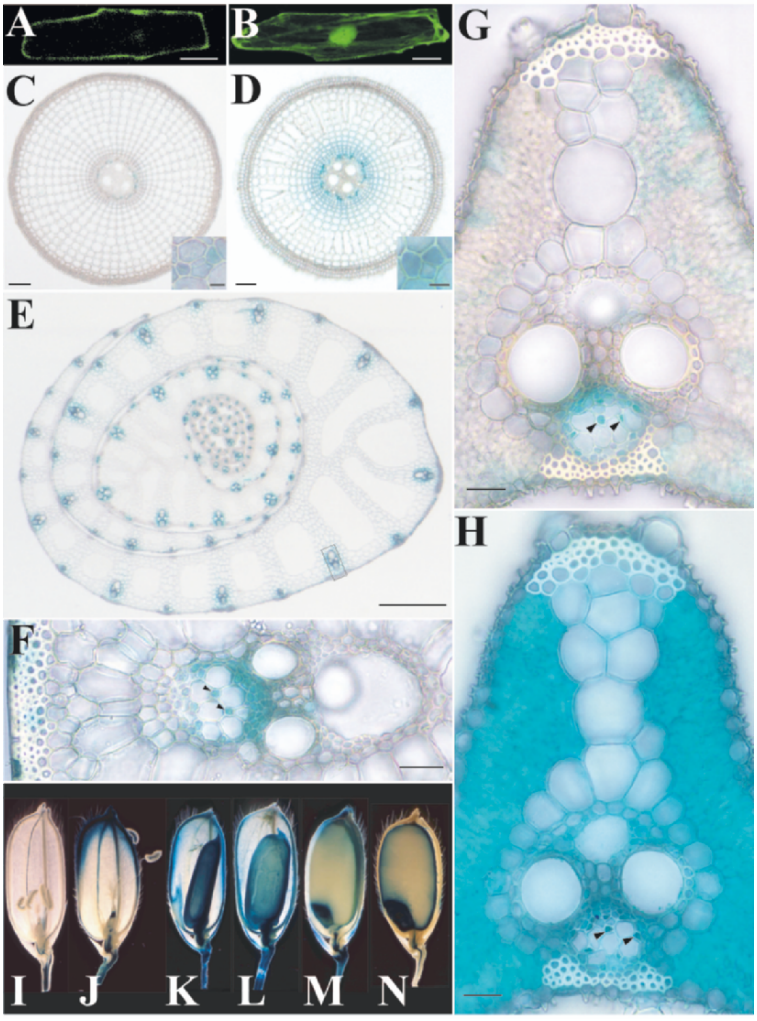

Figure 20-7. The subcellular localization of OsYSL2 and the cell-type-specific localization of *OsYSL2* expression (original figure: Koike *et al.*, 2004). **(A)** and **(B)** Subcellular localization of OsYSL2-GFP fusion **(A)** and GFP **(B)** proteins in onion epidermal cells. **(C)-(N)** *OsYSL2* promoter-*GUS* expression in transgenic rice plants: **(C)** Fe-sufficient roots and **(D)** Fe-deficient roots. Each inset shows a magnified view of phloem cells in the central cylinder; **(E)** Vascular bundles of leaf sheaths; **(F)** One enlarged vascular bundle of leaf sheaths; **(G)** Fe-sufficient leaves and **(H)** Fe-deficient leaves. Arrows indicate companion cells in which expression was obvious. **(I)-(N)** Developing seed during maturation. Before anthesis **(I)**, after fertilization **(J)**, and at 5 **(K)**, 8 **(L)**, 20 **(M)**, and 30 **(N)** days after fertilization. Scale bars = 500 µm for **(E)**; 100 µm for **(C)** and **(D)**; 50 µm for **(A)** and **(B)**; 25 µm for **(F)-(H)**, and 5 µm for insets in **(C)** and **(D)**.

IDS1 is thought to be induced for the purpose of detoxifying metals like Cu and Zn, which are accumulated in Fe-deficient roots. On the other hand, plants often exhibit symptoms of "induced Fe-deficiency" when grown under excess concentrations of other metals such as Mn, Zn, Cu, and Ni (Foy *et al.*, 1998; Alam *et al.*, 2000; Pedler *et al.*, 2000; Patsikka *et al.*, 2002). This has been attributed to competition between Fe and other metals during the uptake process, although similar competition may also occur during translocation and utilization.

We observed an interesting 'mitigation effect' of Fe-deficiency symptoms in tobacco plants grown under a combined deficiency of Fe and other micronutrients (Kobayashi *et al.*, 2003b). Tobacco plants grown under these conditions showed better growth and higher chlorophyll concentrations, as well as reduced levels of Fe-deficiency-induced expression of the *HvIDS2* promoter, than did plants grown in media deficient only in Fe, even though the measured Fe concentration in plant tissues was essentially the same. This phenomenon is likely to be the opposite to the "induced deficiency" described above. Through physiological dissection of these plants, we deduced that this effect could be explained in part by the reduction of chelate competition, suggesting the importance of chelating molecules inside the plant body in metal homeostasis (Kobayashi *et al.*, 2003b).

In graminaceous plants, Zn is thought to be taken up as free Zn(II) ions (Halvorson and Lindsay, 1977), while the Zn(II)-MAs complex has also been proposed as a possible form for Zn uptake (Welch, 1995; von Wirén *et al.*, 1996). A recent study by Suzuki *et al.* (2004) showed that Zn deficiency in barley increased the expression of the genes for the synthesis of MAs, as well as MAs secretion.

2.4.4 Genes involved in the storage of iron

Plants must adapt not only to Fe deficiency but also to Fe overload, because excess Fe can cause oxidative stress to cells through a radical reaction known as the Haber-Weiss or Fenton's reaction (Guerinot and Yi, 1994). Fe overload induces an accumulation of ferritin, an ubiquitous protein for Fe storage in plants, animals, fungi, and bacteria (Briat and Lobréaux, 1997). Ferritin is a multimeric protein whose 24-polypeptide assembly can store up to 4,500 atoms of Fe in a soluble, nontoxic, and bioavailable form. Plant ferritin (phytoferritin) expression is mainly regulated at the transcription level by Fe abundance and is also affected by oxidative stress, abscisic acid, and developmental control. The overexpression of ferritin in transgenic tobacco led to the induction of the root Fe(III)-chelate reductase, which is a typical Fe-deficiency-induced response in non-graminaceous

plants, possibly owing to enhanced Fe sequestration (Van Wuyswinkel *et al.*, 1999). Various studies have also suggested the conserved importance of ferritin in graminaceous species (Lobréaux *et al.*, 1992; Goto *et al.*, 1999; Petit *et al.*, 2001).

Nicotianamine may also play a role in Fe detoxification, as its Fe complexes are thought to be relatively poor Fenton reagents (von Wirén *et al.*, 1999). Immunocytochemical observations of NA showed that Fe-loaded pea plants had increased NA concentrations in the vacuole, possibly sequestering and detoxifying excess Fe (Pich *et al.*, 2001).

2.5 Genes involved in the regulation and signaling of iron status

2.5.1 Iron sensing and possible transcription factors

The mechanism regulating Fe metabolism in higher plants is poorly understood. In non-graminaceous plants, the Fe-deficiency responses in roots are thought to be regulated by both shoot-borne signals and Fe availability at the rhizosphere or the root itself (Schmidt, 2003; Vert *et al.*, 2003). However, the actual signal substances have yet to be identified. Recently, a gene encoding a bHLH transcriptional regulator (*FER*) was isolated using map-based cloning of the tomato Fe-inefficient mutant T3238*fer* (Ling *et al.*, 2002). The tomato *FER* gene is constitutively expressed in roots, suggesting the possible existence of post-translational mechanisms. An *Arabidopsis* homolog of the *FER* gene (*AtbHLH29/FIT1/FRU*) has been recently shown to mediate Fe-deficiency-induced expression of various genes, including *AtFRO2* and *AtIRT1* in *Arabidopsis* roots (Colangelo and Guerinot, 2004; Jakoby *et al.*, 2004).

The molecular mechanism regulating the expression of Fe-deficiency-responsive genes in graminaceous plants is even less well understood, despite the number of genes that have been isolated and characterized. Although we have found some transcription factors induced by Fe deficiency (Negishi *et al.*, 2002; Itai *et al.*, 2004; Ogo *et al.*, 2004), the actual roles of those transcription factors remain unclear. Also, close homologs of the *FER* gene have not been found in graminaceous plants.

2.5.2 Heterogeneous expression of barley iron-deficiency-inducible, root-specific promoters in rice

To clarify the expression mechanism, we analyzed the heterogeneous expression of barley Fe-deficiency-responsive promoters in transgenic rice. The introduction of Fe-deficiency-responsive and root-specific barley

HvNAS1 and *HvNAAT* promoters into rice led to Fe-deficiency-responsive expression both in roots and in leaves (Higuchi *et al*., 2001a; Takahashi *et al*., 2001). Furthermore, the *HvIDS3* promoter also conferred Fe-deficiency-responsive expression strongly in rice roots and moderately in leaves, despite the fact that rice lacks *IDS3* homologs (Kobayashi *et al.*, 2001, 2004). These results suggest a conserved mechanism of Fe-deficiency-induced expression among genes and species, along with some differences in spatial patterns of expression between barley and rice.

3. IDENTIFICATION AND CHARACTERIZATION OF THE IRON-DEFICIENCY-RESPONSIVE *CIS*-ACTING ELEMENTS IDE1 AND IDE2

As summarized in the previous section, the regulation of Fe-deficiency-induced expression is one of the least characterized components of Fe homeostasis in plants. In particular, no Fe-deficiency-responsive *cis*-acting elements have been identified in higher plants. The identification of *cis*-acting elements is a basic approach towards clarifying the regulation mechanisms, and thus we analyzed the barley *IDS2* gene promoter and identified two novel *cis*-acting elements, iron-deficiency responsive element 1 (IDE1) and IDE2 (Kobayashi *et al.*, 2003a). This section summarizes the process of identifying IDE1 and IDE2, along with general aspects of related *cis*/*trans* mechanisms regulating Fe-deficiency-induced expression. The findings are especially valuable for developing new approaches to produce Fe-deficiency-tolerant plants.

3.1 Background

3.1.1 Previously found *cis*-acting elements regulating iron-deficiency-inducible expression

Although no Fe-deficiency-responsive *cis*-acting elements have been identified in higher plants, precise characterizations of *cis*/*trans* mechanisms involved in Fe deficiency-induced gene expression have been conducted in Gram-negative bacteria, yeast, and vertebrates. The ferric iron uptake repressor (Fur) box of Gram-negative bacteria, consisting of a 19-bp inverted repeat (GATAATGATAATCATTATC), was identified as the binding site of the *Escherichia coli* Fur protein, which represses the expression of operons determining siderophore biosynthesis and virulence genes under Fe-sufficient conditions (de Lorenzo *et al.*, 1987; Straus, 1994). In the yeast *Saccharomyces cerevisiae*, the AFT1 consensus binding site

(PyPuCACCCPu) is present in the promoters of various genes regulating Fe and Cu utilization (Yamaguchi-Iwai *et al.*, 1996; Foury and Talibi, 2001). The AFT1 protein binds to this site under conditions of Fe deficiency to promote expression. On the other hand, vertebrates post-transcriptionally regulate the genes involved in Fe homeostasis *via* the *cis*-acting secondary structure of the mRNA sequence called the iron-responsive element, or IRE (Theil, 1990; Klausner *et al.*, 1993). The IREs occur in the 5'-untranslated regions of Fe-induced genes, including ferritin, and in the 3'-untranslated regions of Fe-deficiency-induced genes, including transferrin receptors. When Fe is scarce, the IRE-binding protein (IRE-BP or IRP) binds to the IRE structure, where it inhibits the translational initiation of ferritin mRNA at the 5'-untranslated region and at the same time protects transferrin receptor mRNA from degradation at the 3'-untranslated region, thus promoting expression. When Fe is abundant, the IRP acts as an aconitase enzyme with its [4Fe-4S] state, losing the binding activity to IRE. There are no reports supporting the presence of these characterized regulation mechanisms in plant genes.

3.1.2 Plant *cis*-acting elements mediating iron-regulated expression

In higher plants, the analysis of ferritin gene expression has been developed into a model to understand the mechanism of Fe-regulated expression (Briat and Lobréaux, 1997; Hell and Stephan, 2003). Phytoferritin genes are regulated mainly at the transcriptional level, in contrast to IRE-based regulation in vertebrates. Two groups of researchers have conducted deletion analyses of phytoferritin gene promoters using transient assay systems, leading to the identification of two types of *cis*-acting elements that derepress the expression of phytoferritin genes *via* Fe loading. Wei and Theil (2000) showed that an 86-bp fragment (Fe regulatory element; FRE) controls the Fe-mediated derepression of the soybean ferritin gene. Petit *et al.* (2001) carried out more precise analyses using maize and *Arabidopsis* ferritin gene promoters. They determined the 14-bp *cis*-acting sequence (iron-dependent regulatory sequence; IDRS) by deletion and mutation analysis of the maize ferritin promoter, combined with gel-retardation assays. The IDRS was found to be conserved in the promoter of the *Arabidopsis* ferritin gene (*AtFer1*), and the *AtFer1* IDRS was shown to be functional both in transient assays with *Arabidopsis* culture cells and in transgenic *Arabidopsis* plants. The IDRS and the FRE were not homologous to each other. Nitric oxide was found to be necessary for mediating the transcriptional regulation by the IDRS (Murgia *et al.*, 2002). On the other hand, the tissue-specific or developmental expression of phytoferritin genes

was suggested to be regulated by other unknown *cis*-acting elements (Tarantino *et al.*, 2003).

The finding that the disruption of a bHLH transcriptional regulator (FER) accounts for the Fe inefficiency of the tomato mutant T3238*fer* (Ling *et al.*, 2002) suggests that the consensus binding site of bHLH transcription factor (E-box; CANNTG) is a candidate for plant *cis*-acting elements regulating Fe-deficiency-responsive expression. However, to date no experimental analyses have been reported as to whether certain E-box sequences in a given promoter possess a function in Fe-deficiency-responsive expression or binding capacity to the FER protein.

3.2 Identification of IDE1 and IDE2

3.2.1 Application of a transgenic tobacco system

In general, analyses of plant promoters are performed either by transient expression or by using stable transformants. As the transient expression method generally requires relatively little time and labor, this method has been applied for dissection of many plant promoters, including phytoferritin promoters. However, this method can usually be used only for explanted plant organs or cultured cells and is thus not suitable for analyzing systemic or whole-plant reactions. Problems with large sample variation or low transformation efficiency are also often encountered when genes are introduced by particle bombardment. We used the transient expression method to analyze the *AtNAS* and *HvIDS3* promoters in tobacco and wheat culture cells and in barley roots (Suzuki *et al.*, 2001; unpublished) and found that the *AtNAS3* promoter possesses a putative ethylene-responsive sequence (Suzuki *et al.*, 2001). However, the expression profiles of the introduced promoters were not highly consistent with the whole-plant expression profiles shown by native or transgenic plants. This discrepancy may be explained in part by the loss of tissue specificity and systemic response to nutrient conditions in cultured cells or explants. Also, the expression of transiently introduced plasmid sequences may not be regulated identically to that of stably transferred genome sequences. Thus, a stable transformation system is thought to be more favorable than transient assay systems for analyzing the properties of Fe-deficiency-responsive promoters, especially for the precise analysis of tissue-specific expression and the response to nutrient deficiencies.

We therefore analyzed the Fe-deficiency-inducible, root-specific expression of the barley *IDS2* gene using a stable transformation system with transgenic tobacco. Tobacco is a dicotyledonous plant whose transformation system was established at an early stage of plant biotechnology. Our overall

analysis consisted of the construction of various promoter fragments fused to the *GUS* gene in a binary vector, the generation of T_1 seeds of transgenic tobacco, the confirmation of transgene insertion, and the enzymatic detection of promoter activity using T_1 transformants under conditions of Fe deficiency and Fe sufficiency. Although this process was labor intensive and time consuming, it was highly suitable for the detection of responses to Fe deficiency. To overcome the large variation in GUS activity between transgenic lines (possibly owing to positional and other effects at gene integration), we analyzed ten to 16 independent transgenic lines for each construct, and the acquired data were statistically evaluated using a non-parametric Mann-Whitney test.

3.2.2 Deletion analysis

To determine the main region(s) of the *IDS2* promoter responsible for specific expression, eight derivatives with 5'-deletions between positions -1696 and -47 (relative to the translation start site) of the *IDS2* promoter were generated and analyzed (Figure 20-8; Yoshihara *et al.*, 2003). Each 5'-deletion construct induced strong GUS activity only in Fe-deficient roots, indicating the conservation of the mechanism that regulates *IDS2* gene expression even in tobacco plants that use a different Fe-acquisition strategy. On the other hand, the deletion constructs did not respond to Mn or Zn deficiency in transgenic tobacco, unlike native expression of the *IDS2* gene in barley (Kobayashi *et al.*, 2003b; unpublished). This suggests differences between barley and tobacco in the endogenous *trans*-acting factors that sense Mn and Zn deficiencies.

The shortest 5'-deletion fragment (construct d8) still showed marked induction in response to Fe deficiency, although substantial changes were observed in the induced activity of some of the deletions. This indicated that the sequence between positions -272 and -47 contains the dominant *cis*-acting elements required for Fe-deficiency-inducible expression (Yoshihara *et al.*, 2003). Furthermore, 3'-deletion analysis between the -1696 and -91 region of the *IDS2* promoter demonstrated that the element(s) within the -272/-91 sequence were not only sufficient but also essential for the specific response to Fe deficiency (Kobayashi *et al.*, 2003a).

At the same time, a similar 5'-deletion analysis of the *HvNAS1* promoter was also conducted using transgenic tobacco (Higuchi *et al.*, 2001b). The *HvNAS1* promoter also induced Fe-deficiency-responsive, root-specific expression in tobacco plants, and the dominant *cis*-acting elements for specific expression were suggested to be situated within 348 bp from the translation start site. However, the corresponding *cis*-acting elements have not yet been experimentally identified.

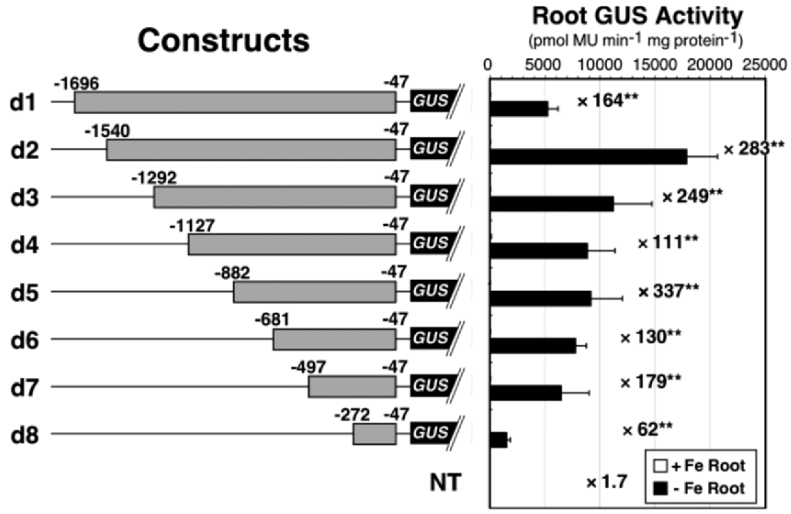

Figure 20-8. The 5'-deletion analysis between positions -1696 and -91 of the *IDS2* promoter (original figure: Yoshihara *et al.*, 2003). Eight sequential 5'-deletion clones that contain promoter regions of the *IDS2* gene were fused to the *GUS* gene and were transferred into tobacco plants. The GUS activity in roots of the T_1 transformants was measured after plants were grown on an Fe-sufficient liquid medium (+Fe) or on a liquid medium without Fe (-Fe) for 30 days. Data are means ± SE of 11-16 independent transgenic lines for each construct or four seedlings of non-transformants (NT). The numerals and asterisks on the right indicate the relative ratio of GUS activity in -Fe roots to the GUS activity in +Fe roots (induction ratio), and significant differences between +Fe and -Fe conditions were analyzed using the non-parametric Mann-Whitney test (*, $p < 0.05$; **, $p < 0.01$). No substantial activity was detected in leaves.

We then produced four 3'-deletion fragments within the -272/-91 sequence of the *IDS2* promoter and fused them to the -46/+8 or -90/+8 region (from the transcriptional start site) of the cauliflower mosaic virus 35S promoter (35SΔ46 and 35SΔ90, respectively; Figure 20-9). Constructs 11-46 and 12-46, carrying the -272/-91 or -272/-136 sequence fused to 35SΔ46, conferred strong GUS expression in roots only under conditions of Fe deficiency. Deletion from -136 to -181 (construct 13-46) markedly reduced the expression in Fe-deficient roots to nearly the basal level. The connection of the *IDS2* promoter fragments to 35SΔ90 instead of 35SΔ46 enhanced the activity either in roots or leaves owing to an enhancer-like effect of the 35S promoter fragment. In this case, the deletions between -91 and -227 had no pronounced effect; the responsiveness to Fe deficiency was significantly retained in the -272/-227 fragment (construct 14-90). These results suggested the presence of two dominant regions containing *cis*-acting elements responsive to Fe deficiency: the -180/-136 region and the -272/-227 region.

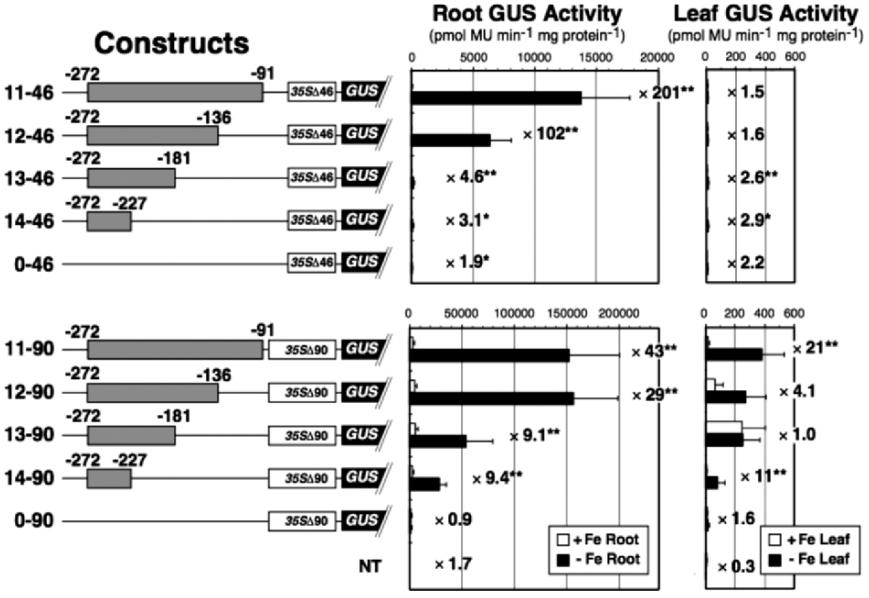

Figure 20-9. The 3'-deletion analysis between -272 and -91 of the *IDS2* promoter (original figure: Kobayashi *et al.*, 2003a). Four sequential 3'-deletion fragments of the -272/-91 region of the *IDS2* promoter were fused upstream from the -46/+8 or -90/+8 region of the cauliflower mosaic virus 35S promoter (35SΔ46 and 35SΔ90, respectively), connected to the *GUS* gene, and analyzed similarly to Figure 20-8. The GUS activities in roots and leaves of T_1 tobacco transformants are shown in the center and to the right, respectively.

3.2.3 Mutation analysis

The site of the *cis*-acting elements was determined by mutation analysis (Figure 20-10). Among the five positions of the 9-bp mutations within the -180/-136 region (Figure 20-10A), mutations within the sequence between positions -180 and -154 (constructs M1-46, M2-46, and M3-46) did not decrease the Fe-deficiency-responsive expression in roots. In contrast, two mutations within the sequence between positions -153 and -136 (constructs M4-46 and M5-46) markedly reduced the responsiveness to Fe deficiency, demonstrating that the *cis*-acting element within the -180/-136 region is situated at -153/-136. This *cis*-acting element was designated IDE1. An internal deletion of the sequence between -226 and -181 (clone N2-46) did not reduce the responsiveness, whereas the 5'-deletion of the whole distal fragment (clone N3-46) completely eliminated GUS expression.

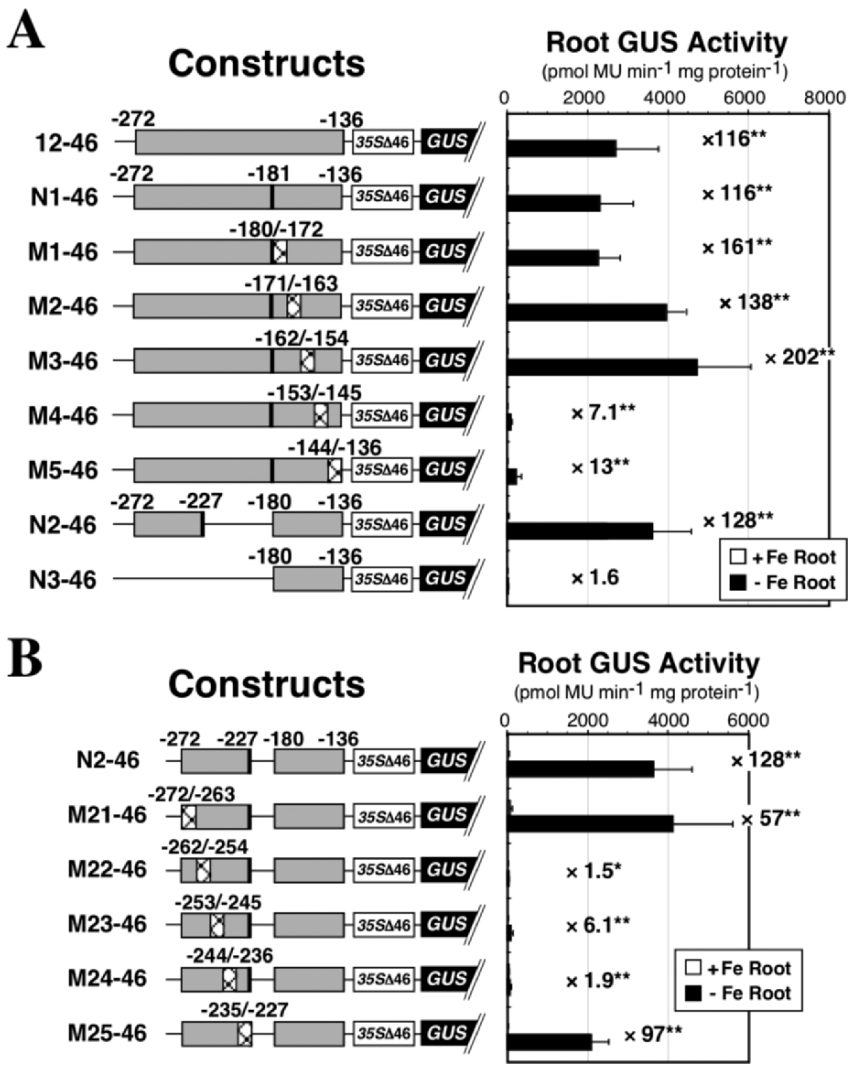

Figure 20-10. Mutation analysis of the -180/-136 region **(A)** and the -272/-227 region **(B)** (original figure: Kobayashi *et al.*, 2003a). Mutations of sequential intervals of 9-10 bp (crosshatched areas) were introduced in the -180/-136 region **(A)** or in the -272/-227 region **(B)**, and the promoter activities were analyzed similarly to Figure 20-8 after seven-days treatment of +Fe or -Fe. Black areas indicate mutated sequences at the fragment junctions. No substantial activity was detected in leaves.

The site of the element within the -272/-227 region was similarly determined using clone N2-46 and mutated derivatives (Figure 20-10B). Mutations at -272/-263 or -235/-227 did not influence the promoter activity.

Conversely, mutations within the sequence between positions -262 and -236 (constructs M22-46, M23-46, and M24-46) almost completely abolished the responsiveness to Fe deficiency, localizing the *cis*-acting element to -262/-236. This *cis*-acting element was designated IDE2.

3.2.4 Properties of IDE1 and IDE2

The synergistic properties of IDE1 and IDE2 are shown in Figure 20-11. When 35SΔ46 was used as a minimal promoter, the deletion or mutation of either IDE1 or IDE2 resulted in a marked or complete loss of responsiveness to Fe deficiency. Therefore, IDE1 and IDE2 synergistically drive Fe-deficiency-inducible expression in the roots. The tissue specificity of the induced expression was histochemically observed (Kobayashi *et al.*, 2003a). Promoter fragments containing IDE1 and IDE2, when fused to 35SΔ46, drove expression mainly in the pericycle, endodermis, and cortex of Fe-deficient roots, resembling the spatial pattern of native *IDS2* expression in barley (Yoshihara *et al.*, 2003).

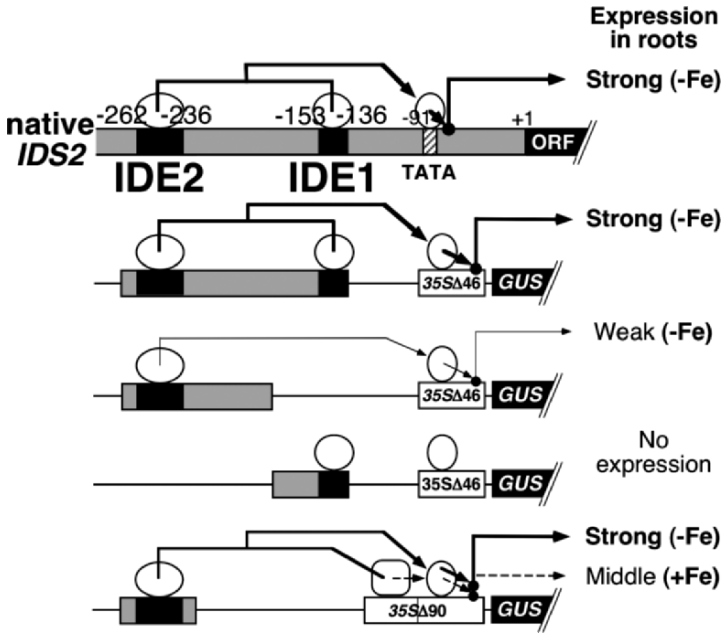

Figure 20-11. Synergistic properties of the two identified *cis*-acting elements (IDE1 and IDE2; original figure: Kobayashi *et al.*, 2003a). IDE1 and IDE2 are the *cis*-acting elements situated at positions -153/-136 and -262/-236 of the *IDS2* gene promoter, respectively. The coexistence of IDE1 and IDE2, or alternatively IDE2 and 35SΔ90, is necessary for inducing strong expression in -Fe roots.

When 35SΔ90 was fused, the Fe deficiency responsiveness conferred by the -272/-227 region, which consists of IDE2 and an additional 19 bp, was obvious in both the presence and absence of IDE1. These constructs expressed GUS strongly in a wide range of tissues, including the epidermis of Fe-deficient roots, which could be explained by synergistic interactions between *IDS2* promoter fragments including IDE2 and the 35SΔ90 fragment that contains an enhancer-like *cis*-acting sequence termed *as-1* (Kobayashi *et al.*, 2003a).

IDE1 and IDE2 did not show any similarity to previously identified *cis*-acting elements involved in Fe homeostasis or to binding sites with bHLH (CANNTG). Therefore, IDE1 and IDE2 are novel *cis*-acting elements that confer responsiveness to Fe deficiency. IDE1 and IDE2 are also the first *cis*-acting elements identified as being responsible for micronutrient deficiency-inducible expression in higher plants.

3.3 Possible conservation of an IDE-mediated regulation mechanism in various genes and species

3.3.1 Sequence comparison of IDE1, IDE2, and other iron-deficiency-inducible promoters

We found many sequences homologous to IDE1 in other Fe-deficiency-inducible gene promoters (Figure 20-12). Of these, the *HvNAAT-A* and *HvNAAT-B* promoters possess conserved sequences strikingly similar to IDE1 and its 3'-flanking 4 bp. Sequences homologous to IDE1 were also found in *HvNAS1*, *HvIDS3*, *OsNAS1, OsNAS2,* and *OsIRT1* promoters, which covered all of the previously identified promoters of barley and rice Fe-deficiency-inducible genes involved in Fe uptake.

Furthermore, recent rice genome database information combined with microarray and Northern analyses revealed that IDE1-like and IDE2-like sequences are present in many other promoters of Fe-deficiency-inducible genes involved in the biosynthesis of MAs, including *OsNAAT1*, *OsFDH*, and *OsIDI1*, as well as a gene encoding methylthioribose kinase (*OsMTK1*) (Figure 20-1, underlined enzymes), suggesting a strong relationship between Fe acquisition processes and their regulation by homologous IDE-like, *cis*-acting elements (Kobayashi *et al*., 2005). IDE1-like and IDE2-like sequences are estimated to be over-represented among promoters of the Fe-deficiency-induced rice genes related to Fe uptake (Kobayashi *et al*., 2005).

Sequences similar to that of IDE1 were also found in some Fe-acquisition-related gene promoters of *Arabidopsis*, including *AtFRO2*, *AtIRT1*, *AtNAS1*, *AtNAS2,* and *AtNAS4* promoters (Figure 20-12). As our recent expression analysis suggests that *AtNAS1* and *AtNAS2* genes are

Fe-deficiency responsive (unpublished), non-graminaceous plants may also share homologous *cis*-acting sequences for Fe-deficiency-inducible gene expression. IDE1-like sequences, but not IDE2-like sequences, may be over-represented among Fe-deficiency responsive *Arabidopsis* promoters related to Fe uptake (Kobayashi *et al*., 2005).

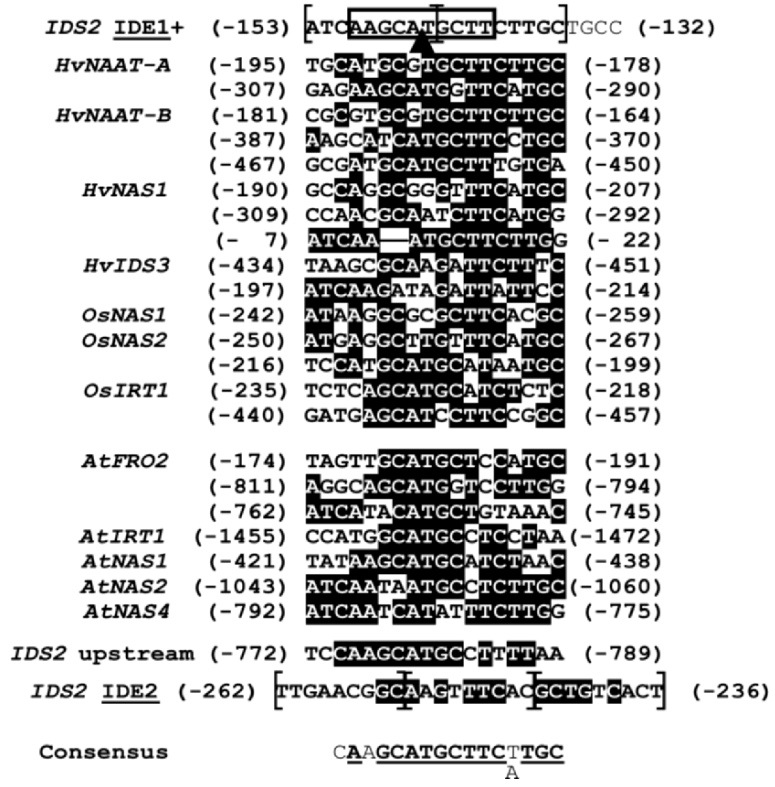

Figure 20-12. Sequence comparison between IDE1, other Fe-deficiency-inducible promoters, and IDE2 (original figure: Kobayashi *et al*., 2003a). *IDS2* IDE1+ represents IDE1 and its 3'-flanking 4 bp, which is strikingly conserved in the *HvNAAT* promoters. The palindromic sequence in IDE1 is indicated by the bold box and arrowhead. Brackets in IDE1 and IDE2 indicate the sites of mutations in linker-scanning analyses. White letters in black areas indicate sequences identical to IDE1+. The distances from the translation start sites are shown in parentheses.

The upstream region of the *IDS2* promoter itself contained a region homologous to IDE1 at -772/-789 (Figure 20-12). In addition, IDE2 was also homologous to IDE1 and its 3'-flanking 4 bp. IDE1 and IDE2 may share functional sequences.

3.4 Iron-deficiency-inducible expression mediated by IDE1 and IDE2 in rice

3.4.1 Attempts to construct super-promoters that induce strong expression in iron-deficient rice roots

In order to apply IDE1 and IDE2 for the production of rice plants that can tolerate Fe deficiency, we aimed to construct super-promoters by combining IDE1 and IDE2 elements with other enhancer-like sequences to increase gene expression in Fe-deficient roots of rice plants (Kobayashi *et al.*, 2004). Several *IDS2* promoter fragments that contained IDE1 and IDE2 were used as modules to construct artificial promoters (Figure 20-13A). These modules were diversely arranged and repeated, or were linked to *IDS3* promoter (Figure 20-13B). Each promoter was fused to 35SΔ90, which enhances expression in tobacco (Figure 20-9), and the tobacco mosaic virus 5' leader sequence (Ω), which is used widely as a translational enhancer. Native promoters of *IDS2* and *IDS3* were also analyzed. Each artificial and native promoter was connected to *GUS* and was introduced into rice. T_1 transformants of 10–12 independent plant lines for each construct were analyzed for GUS expression. All artificial and native promoters clearly responded to Fe deficiency, inducing extremely strong expression in roots and moderate expression in leaves (Figure 20-13B). The artificial promoters also drove substantial gene expression in Fe-sufficient roots and leaves, whereas the native promoters induced relatively low expression under the same conditions. Each of the five artificial promoters produced an essentially identical pattern of gene expression. Repetitions of the promoter elements did not drive stronger expression than that driven by the single-copy artificial promoter or that driven by the native promoters in Fe-deficient roots. It is possible that one copy each of IDE1 and IDE2 is sufficient for gene function. Alternatively, *trans*-acting factors or signaling pathways may be the limiting factors for strong gene expression in rice roots (Kobayashi *et al.*, 2004).

3.4.2 Expression patterns mediated by IDE1 and IDE2 are conserved among rice genes involved in MAs synthesis

Although we were unable to construct 'super' promoters that induce even greater expression than do the native *IDS2* or *IDS3* promoters under conditions of Fe deficiency, we did acquire valuable information about gene expression mechanisms through this analysis. Importantly, we found that one copy each of IDE1 and IDE2 is sufficient to induce strong Fe-deficiency-responsive gene expression in rice (Figure 20-13B). Synergistic

effects of the combination of IDE1, IDE2, and 35SΔ90 in artificial promoters would have led to a relatively greater level of basal expression in Fe-sufficient plants and to gene expression that was dominant in vascular and exodermal tissue (Kobayashi *et al.*, 2004).

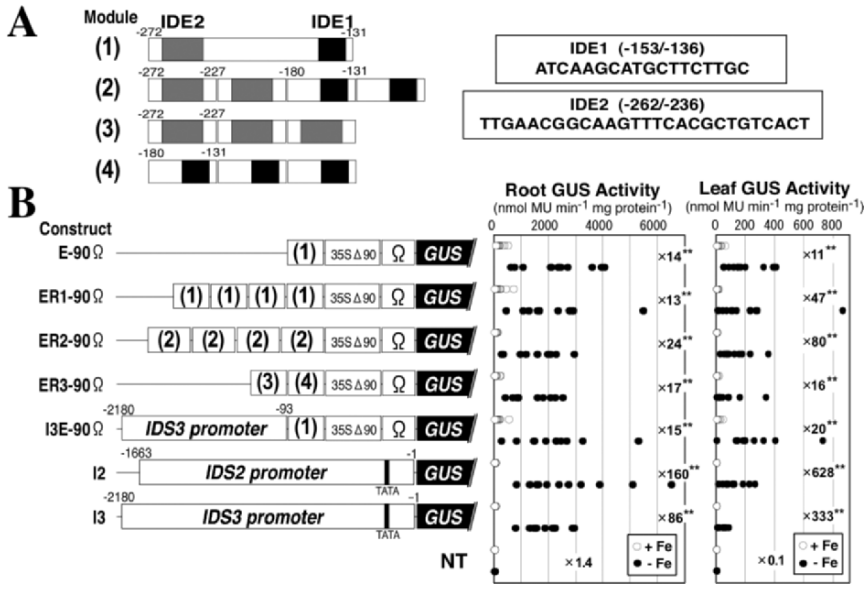

Figure 20-13. Construction of artificial promoters and the GUS activity in transgenic rice plants (original figure: Kobayashi *et al.*, 2004). **(A)** Modules of *IDS2* promoter fragments containing IDE1 and IDE2 that were used to construct artificial promoters. **(B)** Constructs introduced into rice and the GUS activity in T_1 transformants. Structures of the promoter–*GUS* constructs are on the left. Several combinations of *IDS2* promoter fragments (1) to (4) in **(A)** were fused upstream of 35SΔ90 and the tobacco mosaic virus 5' leader sequence (Ω), and were connected to the *GUS* gene (constructs E-90Ω, ER1-90Ω, ER2-90Ω, ER3-90Ω, and I3E-90Ω). The native barley *IDS2* or *IDS3* promoter was fused upstream of *GUS* (constructs I2 and I3). GUS activities in roots and leaves of T_1 rice transformants are in the center and to the right, respectively. Between ten and 12 independent lines for each construct or seven non-transformants (NT) were grown under +Fe (open circle) or -Fe (closed circle) conditions for seven days. Each dot represents the activity of each transgenic line. The other notes are the same as for Figure 20-8.

The expression analysis of native *IDS2* and *IDS3* promoters also provided novel findings. Expression was highly Fe-deficiency-specific and was dominant in the roots (Figure 20-13B), confirming the expression pattern observed by the introduction of a barley genomic fragment containing the *IDS3* promoter (Kobayashi *et al*., 2001). Furthermore, histochemical staining revealed that expression was dominant within vascular bundles and the root exodermis (Kobayashi *et al.*, 2004). These expression profiles were highly

homologous to those of the *OsNAS1*, *OsNAS2*, and *OsNAAT1* promoters (Figure 20-3; Inoue *et al.*, 2003, 2004). This supports the view that a common mechanism regulates the expression of graminaceous genes involved in Fe acquisition.

As reviewed so far, the majority of rice genes involved in Fe uptake, as well as the promoters of barley genes involved in MAs synthesis that were heterologously introduced into rice, exhibit strong responses to Fe deficiency in the roots and moderate responses in the leaves. Since many of these promoters contain IDE-like sequences, rice would possess regulation mechanisms that induce gene expression in response to Fe deficiency in the leaves as well as in the roots, and IDE-like sequences and related *trans*-acting factors may be at least partially responsible for these regulation mechanisms.

4. FUTURE PERSPECTIVES TOWARD UNDERSTANDING THE MECHANISMS AND FACILITATING THE PRODUCTION OF IRON-DEFICIENCY TOLERANT PLANTS

The molecular components reviewed in this chapter are linked to each other as shown in Figure 20-14. Globally, graminaceous plants are extremely important staple crops; therefore, many efforts have been made to enhance agriculturally favorable traits. Takahashi *et al.* (2001) successfully produced rice plants with an enhanced tolerance for low Fe availability by introducing barley genomic fragments containing *HvNAAT* genes and their native promoters. We further introduced several barley genes involved in MAs biosynthesis into rice, conferring some tolerance to Fe deficiency (Nakanishi *et al.*, 2002; Mori, 2002). To additionally enhance Fe-deficiency tolerance, however, components other than those involved in the biosynthesis of MAs may require manipulation as well. The fortification of the Fe content of seeds is another important challenge in this field. Goto *et al.* (1999) produced rice plants with an Fe content of T_1 seeds as much as threefold greater than that of non-transformants by introducing a soybean ferritin gene under the control of the seed-specific promoters of rice glutelin. Although an enhancement of the Fe-storage capacity in the sink would by itself lead to an induction of the Fe uptake system, recent studies suggest that the concomitant enhancement of Fe uptake and/or translocation is necessary for further Fe fortification (Qu *et al.*, 2005).

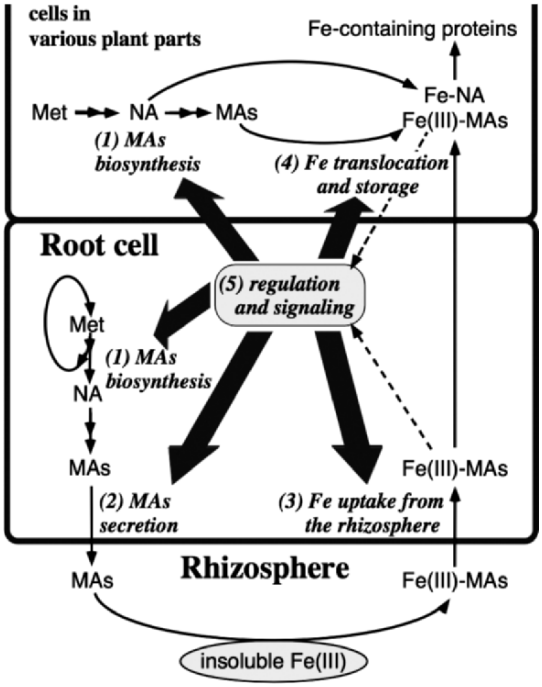

Figure 20-14. The linkage among the molecular components reviewed in this chapter. Solid lines represent movements of substances involved in Fe utilization, and broken lines represent unknown signals sensing Fe availability; bold gray arrows represent expressional regulation of each molecular component.

Thus, the further characterization of Fe homeostasis, especially with regard to poorly understood components such as the secretion mechanism of MAs and metal translocation, and a comprehensive understanding of the plant functions involved in each component will become increasingly important for future advances in this field and for the production of plants that are tolerant to Fe deficiency or that have a high Fe content. The application of the PETIS method to novel transporter mutants will aid in understanding translocation mechanisms. Regulation and signaling, which link the overall response to Fe availability, are also of particular importance (Figure 20-14). The identification of IDE1 and IDE2 marks a milestone toward clarifying the mechanisms of regulation at the molecular level; we are currently attempting to isolate *trans*-acting factors that interact with IDE1 or IDE2. Methods such as mutant screening will be helpful in understanding regulation. Manipulation of the *trans*-acting factors and *cis*-acting elements involved in Fe metabolism will become an attractive

approach for producing crops that are even more tolerant to Fe deficiency and that possess other favorable traits.

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Chapter 21

APPLICATION OF STABLE ISOTOPES IN PLANT IRON RESEARCH

ANA ÁLVAREZ-FERNÁNDEZ
Departamento de Nutrición Vegetal, Estación Experimental Aula Dei, Consejo Superior de Investigaciones Científicas (CSIC), Apdo. 202, 50080 Zaragoza, Spain. E-mail: ana.alvarez@eead.csic.es

Abstract: Stable isotopes offer an excellent and safe tool to study uptake and metabolic processes in Fe plant research. Iron stable isotopes (^{54}Fe, ^{56}Fe, ^{57}Fe and ^{58}Fe) can be used either in fractionation or in tracer studies. Although fractionation studies based on Fe isotopic distribution in natural materials have been scarce, valuable information has been already obtained. Iron isotopic fractionation could document Fe transport by organic molecules, microorganisms and possibly plants in the geologic past. Iron tracer studies have been carried out so far in plant research using radioisotopes (especially ^{59}Fe and ^{55}Fe). These studies have been focused on the uptake and breakdown of synthetic or natural chelates, as well as on the shoot translocation rates of the Fe supplied by those compounds. The trend in the coming years for plant Fe nutrition research will be to shift away from high-risk Fe radioisotopes, and use instead enriched Fe stable isotopes, since the analytical techniques used for their quantification are improving progressively. Also, Fe isotope costs are becoming more affordable. Different possibilities to use stable isotopes in Fe plant studies are proposed, in an attempt to suggest new ways to study Fe deficiency in plants. Furthermore, the possible use of other stable isotopes, such as ^{13}C, ^{15}N and others, in plant Fe studies is discussed.

Key words: iron; iron deficiency; plant; stable isotopes; tracers

1. INTRODUCTION

The various isotopes of a given element differ from each other just in the number of neutrons in their atomic nucleus, and therefore have different atomic masses. Isotopes are denoted in symbolic form by using the number of nucleons (protons plus neutrons) as a superscripted prefix to the chemical

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symbol of the element (e.g. ^{12}C and ^{13}C, ^{56}Fe and ^{57}Fe). Isotopes of a given element can be stable or radioactive. Stable isotopes are those forms that do not change over time, whereas radioactive isotopes decay with time by emitting radiation. Most elements occur in nature as several stable isotopes, whose relative proportions can vary substantially depending on the element. For example, 92% of the total Fe occurs as ^{56}Fe, but three other different stable Fe isotopes also occur in nature in much smaller proportions (Adelstein and Manning, 1995). Conversely, Cd has eight different isotopes, with the most abundant accounting only for 28% of total Cd.

In scientific studies, including plant research, stable isotopes can be used in two different approaches. The first approach consists of fractionation studies, based on the natural isotopic distribution of a given element. Isotopic fractionation is a change in elemental isotopic distribution, appearing as a consequence of physical, chemical and biochemical processes that occur at different rates due to the different atomic masses of the isotopes. Therefore, the final isotopic composition of the reaction product can be different from those of the starting reagents. Since the stable isotopes will not decay over time, the stable isotope composition of any material can be used as a signature (also called fingerprint or natural chemical marker). For instance, the $^{13}C/^{12}C$ ratio can be used to distinguish plant materials coming from C_3 and C_4 plant species, since the main C-fixation enzymatic reactions in these two plant strategies, Rubisco (ribulosebisphosphate carboxylase) and PEPCase (phosphoenolpyruvate carboxylase) in C_3 and C_4 species, respectively, have a different degree of C isotope discrimination (Lajtha and Marshall, 1994). The second approach consists of tracing studies, using man-made compounds enriched in a specific isotope. An increasing number of compounds enriched in particular stable isotopes are being produced, facilitating this approach. Both approaches permit to follow the natural cycles of a particular element and to study metabolic processes in which the element is involved. For instance, the mechanisms of action of different fertilizers can be studied using these methods.

Iron is abundant in the earth's crust and is essential for both animals and plants. However, Fe deficiency is a major agronomical problem in large parts of the world (Abadía *et al.,* 2004). Since the mechanisms for plant uptake and transport of Fe are not completely understood, the use of stable isotopes can be an excellent study tool. This chapter covers different possibilities to use stable isotopes in plant Fe research, in an attempt to suggest new ways to study Fe deficiency in plants.

2. IRON ISOTOPES

Native Fe is a mixture of four stable isotopes: ^{54}Fe, ^{56}Fe, ^{57}Fe and ^{58}Fe. The natural abundances of these isotopes range from approximately 92% to less than 1% (Table 21-1). Currently, materials enriched in the four Fe isotopes are available from the Oak Ridge National Laboratory (ORNL; see 4th column in Table 21-1). Other Fe isotopes, such as those with atomic masses of 52, 53, 55, and 59-61 are radioactive and unstable.

Table 21-1. Stable Fe isotopes: atomic masses, natural abundances, and enriched stable isotopes available from the ORNL.

Isotope	Atomic mass[a]	Natural abundance (%)[b]	Enrichment (%)[c]
^{54}Fe	53.9396147	5.845	97.29
^{56}Fe	55.9349418	91.754	99.93
^{57}Fe	56.9353983	2.119	92.44
^{58}Fe	57.9332801	0.282	82.12

[a]Coplen *et al.* (2002). [b]IRMM-014 elemental Fe as reported by Rosman and Taylor (1998). [c]Representative enriched materials available from ORNL.

Iron has a characteristic isotopic signature (Figure 21-1), which is present in any compound containing Fe. This signature could be analyzed with Fe analysis techniques such as inductively coupled plasma spectroscopy-mass spectrometry (ICP-MS) and others. Analytical techniques recently developed, such as time of flight-mass spectrometry [MS(TOF)], have enough mass resolution to allow detection of the Fe isotopic signature in Fe-containing molecules with small molecular mass, therefore permitting their direct identification. However, if the Fe-containing compound has a large molecular mass, the isotopic signature of other elements may mask the Fe signature, making more difficult its identification. The theoretical isotopic signatures of some Fe-containing, small molecules important in Fe uptake and transport in plants are shown in Figure 21-1 B-E: Fe(III)OH-citrate (Figure 21-1B), Fe(III)-mugineic acid (Figure 21-1C), Fe(III)-ethylenediaminetetra-acetic acid (Fe-EDTA; Figure 21-1D) and Fe(II)-nicotianamine (Figure21-1E). All of them clearly show the Fe signature. The usefulness of the metal isotopic signatures as an identification tool is evident when comparing the very different signatures of the complexes of the metal-chelator nicotianamine with Fe(II) and Zn(II) (Figures 21-1E and 21-1F).

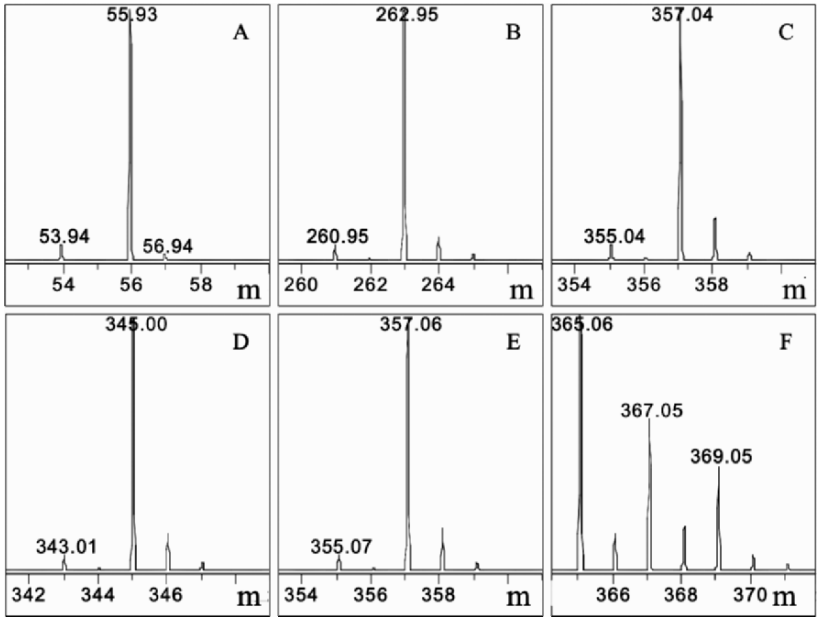

Figure 21-1. Theoretical isotopic mass (m) spectra of Fe (A), Fe(III)OH-citrate (B), Fe(III)-mugineic acid (C), Fe(III)-ethylenediaminetetraacetic acid (Fe-EDTA; D), Fe(II)-nicotianamine (E) and Zn(II)-nicotianamine (F). Fe(III)OH-citrate is the major Fe-citrate species considered to be present in the apoplast of field-grown pear (López-Millán *et al.*, 2001).
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The changes in Fe isotope abundances are usually assessed by comparison to a standard reference material. In the case of Fe, the relative Fe isotope ratio of the mole fractions of ^{56}Fe and ^{54}Fe ($n(^{56}Fe)/n(^{54}Fe)$) is commonly expressed in δ (delta) notation. δ is calculated as follows:

$$\delta^{56}Fe(‰) = [(n^{56}Fe/n^{54}Fe)_{sample} - (n^{56}Fe/n^{54}Fe)_{reference}]/(n^{56}Fe/n^{54}Fe)_{reference} \times 1000$$

For Fe, the standard reference material is IRMM-014 elemental Fe, which has been assigned an exact $\delta^{56}Fe$ value of 0‰ (Rosman and Taylor, 1998). Values of $\delta^{56}Fe$ between –2.9 and +1.36‰ have been found in nature in different materials (Coplen *et al.*, 2002). In both plants and animals $\delta^{56}Fe$ is approximately –2.9‰, which indicates that biological materials are depleted in the heavier ^{56}Fe isotope. Conversely, non-biological materials, such as igneous and sedimentary rocks (Fe-Mn oxides, Fe oxyhydroxide, etc) and groundwater have higher (less negative, closer to 0) $\delta^{56}Fe$ values (Coplen *et al.*, 2002).

Materials enriched in any Fe stable isotope can be used as tracers in biological research. For instance, tracers enriched in ^{54}Fe, ^{57}Fe and ^{58}Fe have been often used in human Fe nutrition research, to study effective Fe absorption and excretion, to develop successful strategies to cure anemia and also in metabolic tracer studies to identify genetic Fe control mechanisms (Wienk *et al.*, 1999; Patterson and Veillon, 2001). Using the same approach, these stable isotopes can be used as tracers in plant research to investigate the uptake, transport and metabolism of Fe. Tracer studies can be developed either using only the Fe relative isotopic composition, by means of metal only-measuring techniques such as ICP-MS, or by studying the real chemical speciation with techniques that can separate and identify the actual Fe-containing compounds, such as HPLC-MS(TOF). The later technique would be particularly useful for the investigation of Fe metabolic pathways. For instance, discerning Fe-containing molecules coming from exogenous sources, such as fertilizers, from others coming from plant and soil native Fe pools would be possible with these methods.

3. USE OF IRON STABLE ISOTOPES IN PLANT IRON RESEARCH

3.1 Fractionation studies

Stable isotope fractionation studies have been traditionally used in geodynamics and material sciences. These techniques are being increasingly used in biological sciences, but few attempts to search for and exploit variations in the abundance of Fe stable isotopes in biological materials have been made. This is due in part to some analytical difficulties. Elements with intermediate masses such as Fe have a lower degree of natural stable isotope fractionation, compared to the larger fractionation occurring in small mass stable isotopes, such as C, O, N and S. Improvements made recently in mass spectrometry techniques for the analysis of Fe isotopes (Crews *et al.*, 1994; Polyakov, 1997; Belshaw *et al.*, 2000) have allowed to find a significant degree of Fe isotope fractionation associated to biological processes (Beard *et al.*, 1999; Brantley *et al.*, 2001; Anbar, 2004).

Plants may produce a measurable Fe-isotopic fractionation because Fe trafficking involves a number of steps, such as reduction by enzymes, transport across membranes, chelation by small organic compounds or proteins, etc. (Marschner, 1995), and these mechanisms may have different rates with different Fe isotopes. Experiments with dissimilatory Fe-reducing bacteria of the genus *Shewanella algae* grown on a ferrihydrite substrate indicate that the $\delta^{56}Fe$ of Fe(II) in solution was isotopically lighter than the

ferrihydrite substrate by 1.3‰ (Beard *et al.*, 1999). The authors indicated that the negative δ^{56}Fe values found in sedimentary rocks could be associated with the biogenic fractionation, and suggested that the isotopic Fe signature may be used to trace the distribution of microorganisms in modern and ancient Earth. More recently, Brantley *et al.* (2001) explained variations in δ^{56}Fe as the consequence of the mechanisms used by bacteria to dissolve Fe from minerals. In order to scavenge Fe, bacteria and plants produce small organic molecules (siderophores and phytosiderophores, respectively) that have large association constants for Fe(III) (von Wirén *et al.*, 2000; Boukhalfa and Crumbliss, 2002). The δ^{56}Fe of Fe dissolved from a silicate soil mineral by siderophore-producing bacteria was 0.8‰ lighter than the bulk Fe in the mineral (Brantley *et al.*, 2001). These data support that bacteria and plants have an isotopically lighter Fe pool than that of Fe-minerals, due to the biological Fe dissolution and uptake processes. A smaller isotopic shift was also observed for the abiotic release of Fe from the silicate soil mineral by two chelates, and the magnitude of the shift increased with the affinity of the ligand for Fe, supporting a kinetic isotope effect during Fe hydrolysis at the mineral surface. Iron isotopic fractionation could therefore document Fe transport by organic molecules or by microorganisms and plants where such entities were present in the geologic past (Brantley *et al.*, 2001).

3.2 Tracing studies

Iron compounds to be used as tracers in plant metabolic studies are obtained by incorporating Fe stable isotopes with low natural abundance, such as ^{54}Fe, ^{57}Fe and ^{58}Fe, into organic or inorganic Fe-containing molecules. The questions to be answered in plant Fe nutrition research are often related to the uptake, transport and homeostasis of Fe. Uptake of Fe can be estimated by determining the fraction acquired by the plant of a Fe stable isotope (or isotopes) given as a tracer. Studies involving metabolic pathways, pool sizes, turnover and nutrient modelling could be carried out.

Iron-tracer experiments in plant Fe nutrition research have been mainly carried out using radioisotopes (specially ^{59}Fe and ^{55}Fe). Studies of the uptake and breakdown of synthetic Fe(III) chelates used to supply Fe to plants were carried out with ^{59}Fe and ^{55}Fe (Tiffin and Brown, 1959; Hill-Cottingham and Lloyd-Jones, 1961; Jeffreys *et al.*, 1961). Both Fe radioactive isotopes have also been used to study the mechanisms of Fe(III)-phytosiderophore uptake by plants (Römheld and Marschner, 1986) and to compare the uptake rates of Fe from different external sources, including phytosiderophores, microbial siderophores and synthetic chelates (Mino *et al.*, 1983; Reid and Crowley, 1984; Römheld and Marschner, 1986; Awad

et al., 1988; Crowley *et al.*, 1992; Duijff *et al.*, 1994). Some of these studies provided evidence for the existence of two different higher plant strategies for the solubilization and uptake of Fe in response to Fe deficiency (Römheld and Marschner, 1986). Iron-59 has also been used to study the shoot translocation of Fe supplied from Fe complexes of siderophores, phytosiderophores and synthetic chelates (Crowley *et al.*, 1992; Duijff *et al.*, 1994; Johnson *et al.*, 2002; Cesco *et al.*, 2004). Preliminary results using positron (γ-ray) ^{52}Fe emission imaging have been obtained to monitor real-time translocation of Fe in barley, rice and maize plants (Nakanishi *et al.*, 2004).

It is evident that Fe stable isotope tracers can also be used in many plant Fe nutrition studies instead of radioisotopes. Difficulties for the use of stable isotopes include the lack of available compounds enriched in stable Fe isotopes, as well as analytical constraints. Nowadays all Fe stable isotopes are commercially available, with different degrees of enrichment and in the metal or oxide forms. Prices are still high but are progressively becoming more affordable (in December 2004, approximately 170-440 US$ per 10 mg, depending on the Fe isotope). Since stable isotopes do not emit radiation, they must be detected by using their exact mass and quantified according to recent mass spectrometry methodological improvements (Crews *et al.*, 1994; Polyakov, 1997; Belshaw *et al.*, 2000; see review by Patterson and Veillon, 2001). Currently, thermal-ionization mass spectrometry (TIMS) and ICP-MS are commonly used (Wienk *et al.*, 1999). Therefore, and following the steps of animal Fe nutrition research, the trend in the coming years in plant Fe nutrition will be to shift away from high-risk radioisotopes and using instead enriched stable isotopes. Iron stable isotopes also have the advantage over the use of Fe radioactive isotopes that they may be traced for extended periods of time, whereas the half-life of radioisotopes (45 days for ^{59}Fe) limits the length of the studies. Multiple-isotope experiments are also easier to conduct using stable isotopes, since problems caused by overlapping radiation energy spectra do not occur (Patterson and Veillon, 2001). The disadvantage of stable isotopes is that they are more expensive than radioisotopes, and also that sample preparation and analytical requirements are more complex.

Very few Fe isotope-containing products are available in the market. Therefore, ^{54}Fe-, ^{56}Fe-, ^{57}Fe- and ^{58}Fe-labelled organic or inorganic compounds must be produced in the laboratory, and there is a lack of published methods for the incorporation of these isotopes into chemical compounds. However, procedures similar to those used to prepare compounds incorporating an Fe radioactive isotope can be followed, with the advantage that radioactivity contamination risks are absent. For example, simple procedures to prepare ^{59}Fe-labelled chelates of siderophores,

phytosiderophores and synthetic chelating agents have been described by Duijff *et al.* (1994) and more recently by Johnson *et al.* (2002).

A major concern in stable isotopes tracing studies is the selection of the isotope to be used and also the dose that should be applied. The essential requirement when using a tracer is that its final concentration must be detectable in the samples. One aspect that must be considered is the increase in the amount of a given isotope necessary to carry out an accurate measurement. Many of these questions have already been answered in human Fe nutrition research, and similar solutions could be found in plant Fe research. Several recent reviews on human nutrition research have discussed the main issues in the field of stable isotopes application for tracing studies (Van Campen and Glahn, 1999; Wienk *et al.*, 1999; Patterson and Veillon, 2001; Fairweather-Tait and Dainty, 2002).

4. OTHER ISOTOPES THAT CAN BE IMPORTANT IN PLANT IRON RESEARCH

Research with stable isotopes has been mainly developed for the study of the natural variations in the stable isotope abundance of light elements such as H, C and O (Belshaw *et al.*, 2000), as well as for tracing metabolic studies using organic molecules enriched in the heavy isotopes of H, C and N (see review by Patterson and Veillon, 2001). Stable isotopes such as ^{2}H, ^{13}C and ^{15}N have been used in some studies related to plant Fe research. The ^{13}C natural abundance was used to study the changes induced by Fe deficiency in C fixation in sugar beet plants (Rombolà *et al.*, 2005). Since Fe-deficient plants have a marked increase in root PEPCase activity (Andaluz *et al.*, 2002), it was hypothesized that an increase in the amount of root C fixation by this enzyme, compared to the amount of C fixed by Rubisco in leaves, would cause higher (less negative) $\delta^{13}C$ values, as a result of the different degree of C discrimination of these two enzymes ($\Delta\delta^{13}C$ of –29‰ for Rubisco and –6‰ for PEPCase; Lajtha and Marshall, 1994). However, $\delta^{13}C$ was instead higher (less negative) in Fe-sufficient than in Fe-deficient plants, and the reason for this finding is still unexplained. In the same study, nutrient solution HCO_3^- was labeled with ^{13}C as a tracer to quantify the contribution of the C deriving from bicarbonate to total plant C (Rombolà *et al.*, 2005). Hydrogen-2, ^{13}C and ^{15}N have also been used to characterize the biosynthetic pathway of mugineic acids, a group of well-known phyto-siderophores produced by graminaceous species in response to Fe stress (Ma and Nomoto, 1994; Ma *et al.*, 1995). Tracing experiments were conducted by feeding roots with several metabolites labelled in different positions with ^{2}H, ^{13}C and ^{15}N. The incorporation of these labelled atoms into mugineic

acids was studied by using NMR (^{1}H, ^{2}H and ^{13}C) and fast atom bombardment (FAB; ^{2}H and ^{15}N) mass spectrometry. From these experiments it was concluded that the methionine recycling pathway was involved not only in ripening processes, but was also required for continued synthesis of mugineic acids in roots.

Carbon radioisotopes such as ^{14}C and ^{11}C have also been used in plant Fe research. Carbon-14 was used to study the plant uptake and breakdown of synthetic Fe(III) chelates by Hill-Cottingham and Lloyd-Jones (1961) and Jeffreys *et al.* (1961), using ^{14}C labelled EDTA and EDDHA to synthesize ^{59}Fe-^{14}C-labeled Fe(III)-EDTA and -EDDHA. These dual-labelled Fe-chelates were used to investigate the uptake of the whole organic molecule by roots (Hill-Cottingham and Lloyd-Jones, 1961). Recently, a protocol to incorporate ^{14}C into nicotianamine, a non-proteinogenic amino acid considered to be a key component in Fe homeostasis in plants, has been developed by Schmiedeberg *et al.* (2003), using the precursor S-adenosylmethionine labelled with ^{14}C and a recombinant nicotianamine synthase. Also, studies with ^{14}C were made to identify maize root exudates involved in Fe trafficking in the rhizosphere and to estimate their amount (Kuzyakov *et al.*, 2003). Maize plants were labelled in a $^{14}CO_2$ atmosphere to separate root-derived and soil-derived organic substances in the rhizosphere using a non-sterile soil, and secreted compounds present in lixiviation fluids were analyzed for total ^{14}C radioactivity and also for composition using pyrolysis field ionization-mass spectrometry. The half-life of mugineic acid secreted by roots of barley grown in $^{14}CO_2$-enriched atmosphere was found to be approximately 24 hours (Mori *et al.*, 1987). Working with barley, positron (γ-ray) ^{11}C emission imaging has been used to investigate whether methionine transported from the shoot was used for the direct synthesis of mugineic acids in the roots of Fe-deficient plants (Nakanishi *et al.*, 1999).

5. FUTURE RESEARCH

Stable isotopes offer an excellent and safe tool to study uptake and metabolic processes in plant Fe research. Important limiting factors for the use of stable isotopes have been, and will be in the short future, the high cost of the enriched Fe stable isotope materials and the availability of ICP-MS and HPLC-MS(TOF) instruments. A variety of experiments may be designed using enriched stable isotopes as tracers. For instance, using two different stable Fe isotopes, such as ^{57}Fe and ^{58}Fe, we can follow the uptake of Fe applied to two different parts of the plant at the same time (e.g. foliar and soil applied), as well as to compare Fe uptake rates from different Fe

chemical sources (e.g. different Fe-chelates applied to the soil). It is also possible to study plant Fe metabolism by using mass spectrometry techniques, such as HPLC-MS(TOF), that allows for the measurement of the isotopic composition of each specific Fe-containing molecule. Other applications could involve investigations on the changes in plant Fe metabolism caused by different factors, such as heavy metals and others. Furthermore, fertilizers labelled with stable isotopes can be used to follow their fate in soil-plant systems, thus obtaining valuable information about fertilizer selection, timing, efficiency and action mechanisms. Finally, stable isotopes may also be applied along with proteomic techniques to study changes in proteins caused by Fe stress, as well as to compare the proteome of plant species with different tolerance to Fe stress, by using recently developed techniques for stable isotope protein labelling (Regnier *et al.*, 2002; Whitelegge *et al.*, 2004). All of these applications of stable isotopes will allow, alone or together with other techniques, a fast progress in Fe plant research in the coming years.

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Chapter 22

GENOMIC RESOURCES OF AGRONOMIC CROPS

SILVIA R. CIANZIO[1], RANDY C. SHOEMAKER[2] and DIRK V. CHARLSON[3]

[1]Department of Agronomy, Iowa State University, Ames, Iowa, USA. E-mail: scianzio@iastate.edu. [2]USDA-ARS, Corn Insect and Crop Genetics Research Unit, Department of Agronomy, Iowa State University, Ames, Iowa, USA. [3]Department of Biochemistry, University of Missouri-Columbia, Columbia, Missouri, USA

Abstract: Iron-deficiency chlorosis in crops grown on calcareous soil is a major agricultural problem. In susceptible genotypes, Fe deficiency causes chlorosis and any yellowness in the foliage will lead to losses in seed yield. The purpose of this chapter is to review genomic information available on Fe efficiency in soybean, maize and rice. A summary of model plants is also presented. In soybean, most of the genomic information on Fe acquisition and uptake was collected during the search for quantitative trait loci (QTLs) for Fe efficiency that could be used in marker-assisted selection. Thirty-six QTLs associated with Fe efficiency have been placed in eight linkage groups (LG), with the greatest density of QTLs on LGs B2 and N. As of recently, functional characterization of any of these soybean QTLs has not been reported. Improvement of Fe efficiency in soybean has been mainly accomplished by classical breeding methods. In maize, 41 gene sequences associated with Fe have been identified. Despite the cloning of some of these genes, improvement in Fe efficiency in maize has been done by using tolerant genotypes in hybrid combinations. To date, and of the three species considered in this review, rice has the largest amount of genomic information available both at molecular and biochemical levels. Genetic transformation in rice has been conducted to improve its Fe efficiency using genes cloned primarily from barley; steps are now being taken to use genomic information from rice itself to improve Fe efficiency through genetic engineering. Field evaluations of rice transformants for Fe efficiency will be required to determine effects of the integrated genes on seed yield, disease resistance, and other important agricultural traits. These evaluations will be mandatory before transgenic cultivars may be used commercially. More genomic information is added every day, and it will greatly increase in all major crops, thereby increasing the practicality of plant transformation in the improvement of Fe efficiency. Traditional breeding will fulfill an important role in this effort, as extensive evaluations will be required

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to assess yield and gene position effects of the newly introduced genes in the plant species of interest.

Key words: iron efficiency; maize; QTL; rice; soybean

1. INTRODUCTION

Maintenance of cellular homeostasis requires sufficient supply of iron (Fe), which is essential to all living organisms (Hell and Stephan, 2003). There is, however, a worldwide Fe-deficiency problem present in numerous crops (Korcak, 1987; Marschner, 1986), farm-produced animals and in humans (Morris, 1987). Fe deficiency does not result from the scarcity of Fe in the environment, but rather from factors that inhibit Fe absorption or impair Fe use in metabolic processes (Welch *et al.*, 1991). Iron-deficiency chlorosis (IDC) of crops grown on calcareous soil, which has high soil pH, is a major agricultural problem, because about 30% of cultivated soils in the world are calcareous (Mori, 1999).

In susceptible genotypes, Fe deficiency causes a characteristic chlorotic phenotype of interveinal chlorosis. Crops harvested for seed will experience yield losses whenever any amount of yellowing occurs (Cianzio, 1991). Fe deficiency also induces less evident changes in roots and shoots that greatly impact the whole-plant metabolism and morphology (Briat *et al.*, 1995; Fox and Guerinot, 1998). Most is known about the effect of Fe-deficiency on root morphology and physiology relative to shoot responses.

At least two distinct root-response mechanisms to Fe deficiency in higher plants have been identified; Strategy I in dicotyledoneous and monocotyledoneous species, with the exception of the graminaceous species, and Strategy II confined to grasses (Marschner and Römheld, 1994). In Strategy I, plants acquire iron resulting from the reduction of Fe^{3+} to Fe^{2+}. In Strategy II, the main step in iron uptake is through chelation (Curie *et al.*, 2001). These processes have been reviewed by Chaney and Bell (1987), Marschner (1986), Marschner and Römheld (1994), and more recently by Schmidt (1999). Up to the mid 1990's, it was thought plants would possess either one of the two Strategies with no detectable overlap. The advent of more sophisticated physiological, biochemical and molecular techniques has provided a wealth of information indicating that the distinction between plants based on the two strategies no longer holds true (Bughio *et al.*, 2002; Charlson and Shoemaker, unpublished data; Suzuki *et al.*, 1999; Varotto *et al.*, 2002; von Wirén *et al.*, 1993).

The purpose of this chapter is to review genomic information concerning Fe-efficiency metabolism in three agronomic crops, soybean, rice, and maize, which constitute important sources of human food. The review refers

to genetic responses related to Fe acquisition by roots and Fe transport into root tissue that take place in plants grown under Fe-limiting or Fe-deficient conditions either in soils or in nutrient solutions. Information about model plants will also be discussed, as it closely pertains to the crops of interest.

2. GENOMIC RESOURCES IN MODEL PLANTS

The work in *Arabidopsis thaliana* as a model plant for Strategy I has contributed an enormous amount of information to the understanding of the Fe uptake process. This has been supplemented with research conducted in *Lycopersicum esculentum* (tomato), and *Pisum sativum* (pea). For Strategy II plants, work has been conducted in *Hordeum vulgare* (barley) and maize, and other grass species.

2.1 Strategy I genes

All plants, except grasses, use Strategy I to acquire Fe by three reactions: (i) initial excretion of protons carried out by a plasma membrane P-type ATPase to acidify the surrounding rhizosphere therefore increasing Fe^{3+} solubility; (ii) reduction of Fe^{3+} to Fe^{2+} completed by a Fe^{3+}-chelate reductase; and finally (iii) transport of Fe^{2+} by transporters across the plasmalemma of root epidermal cells (Hell and Stephan, 2003; Fox and Guerinot, 1998). It has been shown that both the Fe^{3+}-chelate reductase and Fe^{2+}-transport activities are enhanced under Fe deficiency (Connolly *et al.*, 2002; Fox and Guerinot, 1998; Robinson *et al.*, 1999; Vert *et al.*, 2002).

In Fe acquisition, genes encoding for Fe^{3+}-chelate reductase have been cloned from *A. thaliana*, *FRO2* (Robinson *et al.*, 1999) and pea, *FRO1* (Waters *et al.*, 2002). Both research groups reported the two genes are up-regulated in roots in response to Fe deficiency. Moreover, *A. thaliana* lines with an inactivated *FRO2* gene show reduced Fe^{3+}-chelate-reductase activity during both Fe-sufficient and Fe-deficient conditions with chlorotic leaves and plant growth impaired on Fe-deficient medium (Robinson *et al.*, 1999).

Once the Fe has been reduced at the root, the next step is transport into the plant. The Fe-transporter genes, *IRT1* and *IRT2*, have been cloned from *Arabidopsis* (Eide *et al.*, 1996; Vert *et al.*, 2001) as well as its orthologs from pea (*RIT1*) (Cohen *et al.*, 1998) and tomato (*IRT1* and *IRT2*) (Eckhardt *et al.*, 2001). The *Arabidopsis* gene *IRT1* has been established as the major Fe-uptake component for acquisition of Fe from soil (Vert *et al.*, 2002). In addition, *Arabidopsis* IRT1 also transports zinc, manganese, and cadmium (Fox and Guerinot, 1998; Guerinot, 2000; Korshunova *et al.*, 1999). *Arabidopsis* IRT2 is also induced in roots of *Arabidopsis* in response to Fe

deficiency (Eide *et al.*, 1996; Vert *et al.*, 2001). Transporters involved in Fe, and other metals have been identified as belonging to the ZIP family of transporters (Eng *et al.*, 1998; Fox and Guerinot, 1998; Guerinot, 2000). Other Fe transporter genes (*Nramp*) have also been cloned from *A. thaliana* (Curie *et al.*, 2000; Lanquar *et al.*, 2004; Thomine *et al.*, 2000) that share important similarity with their mammalian counterparts. Although their role is not yet completely clear, at least *NRAMP* 1, 3, and 4 seem to be induced by Fe deficiency, and are expressed in roots and leaves of *A. thaliana*. It is thought they might contribute to Fe homoestasis in addition to IRT transporters (Curie *et al.*, 2000; Thomine *et al.*, 2000). An additional transporter gene, *FRD3*, has been recently identified in *A. thaliana* (Rogers and Guerinot, 2002). The authors hypothesize that FDR3 is involved in transporting a low-molecular weight iron chelator into the xylem. The reader is referred to Chapter 16 on FRD3's role in iron homeostasis.

2.2 Strategy II genes

Grasses and important food plants such as rice (*Oryza sativa)* and maize (*Zea mays*) use Strategy II for Fe acquisition. Roots release (i) phytosiderophores (PSs) that chelate Fe^{3+} in the rhizosphere, and (ii) specific plasmalemma transporter proteins import the Fe^{3+}-PS complexes into the plant (Römheld and Marschner, 1986). Both processes are enhanced in response to Fe deficiency via up-regulation of the underlying genes (Hell and Stephan, 2003) indicating their importance in Fe acquisition.

Most of the underlying genes for Strategy II have been cloned from barley and rice, and Mori has scholarly reviewed the topic (1999). Fe chelation was recognised when plant PSs were first identified by Takagi in 1976 (Mori *et al.*, 1991). PSs belong to the mugineic acid (MA) family of chelators, solubilizing inorganic Fe^{3+} compounds by chelation, and the Fe-PS complexes are taken into the root cells by a specific transport system of the plasma membrane (Mori, 1999). Biosynthesis of MA starts from three molecules of methionine from the Yang cycle that are integrated into one molecule of nicotianamine (NA) in an enzymatic step catalyzed by nicotianamine synthase (Hell and Stephan, 2003). NA synthase has been cloned from tomato, barley, and rice; and its expression in roots is strongly up-regulated by Fe availability. Formation of MA and MA derivatives, as well as the Strategy II mechanism, has been summarized by Hell and Stephan (2003).

A Fe-PS transporter has been identified in Strategy II plants. Uptake of Fe^{3+}-PS complex has been clarified by Curie *et al.* (2001) who cloned the mutant gene of the transport-defective *ys1* (*yellow stripe1*) maize mutant. The wild type *YS1* gene complements this maize mutant and is up-regulated

in roots and shoots. In addition, Roberts *et al.* (2004) have shown in yeast cells defective in Fe transport that maize YS1 facilitates the transport of Fe^{3+}-MA; however, YS1 also transports Fe^{2+}-NA. Due to the ubiquitous distribution of NA in the plant kingdom and across Fe-acquisition strategies, NA has been suggested as a substrate for Fe transport within the plant. This hypothesis has been further supported by the identification of eight *YS1*-like genes in the MA-free dicot *A. thaliana* (Curie *et al.*, 2001) and *YS1*-like genes identified from ESTs (expressed sequence tags) of soybean, *Medicago*, potato, and tomato (Charlson and Shoemaker, unpublished data). Several YS1-like transporters in *Arabidopsis* have been shown to transport Fe-NA complexes, suggesting that NA serves as a ligand for the transport of Fe and possibly other metals (E. Walker, University of Massachusetts; personal communication cited by Hell and Stephan, 2003).

2.3 Future use of plant models

Important implications may be drawn from the summary presented, both for Strategy I and Strategy II plant models, which refer to numerous similarities in the Fe acquisition process across all living organisms. Additional information is also derived from research conducted in *Saccharomyces cerevisiae* and mammals. The conservation and repetitive steps in the process of Fe uptake across plant species support the usefulness of model organisms, and much more importantly, provides rationale for the possible use of genes to genetically improve Fe acquisition across plant species. The possibility of producing transgenic plants, in which some of these genes may be used to improve Fe uptake, opens up the traditional field of breeding and plant improvement into a new frontier.

3. GENOMIC RESOURCES IN AGRONOMIC CROPS

Improvement of Fe nutrition in crops may be considered from two different perspectives, equally important, although distinct in objectives. One is to improve Fe acquisition and uptake by the plant, thereby diminishing Fe-deficiency chlorosis (IDC) in crops. Improving Fe acquisition will increase crop yields and expansion of plantings to geographical areas previously not optimal for maximum seed and fruit yields. The second perspective is modification of Fe-storage proteins in seeds to increase bioavailable Fe. This would alleviate Fe malnutrition directly in human populations, particularly children and young women, by providing more nutritious plant-derived foods. However, breeding for Fe

uptake alone may not necessarily lead to increases in Fe concentration and/or bioavailability in the seed. Possibly the two Fe problems may have to be treated independently, because acquisition occurs at the root level and increases of seed Fe concentration involves efficient loading into the phloem and storage in seeds. Although different approaches may be needed, successful completion of these two objectives would meet human requirements for improving Fe-efficiency and nutritive qualities in crops.

Currently, the most efficient manner in which to overcome IDC in economically important plant species is to modify plant genetics through plant breeding. If plants are bred for adaptation to high lime soils in which Fe is not directly available to them, yields will be increased. During the late 20th century this was done using classical breeding approaches (Cianzio, 1991). Classical breeding first required the identification of genotypes resistant to IDC. These were crossed with susceptible genotypes possessing other desirable traits. The segregating populations developed from such crosses allowed for the selection of IDC-resistant genotypes possessing agronomically-important traits.

The breeding outline just described is, however, an oversimplification of a complicated and long process, in which years of screening under controlled and field conditions are necessary to cope with the inherent variation of Fe distribution in the soil, the interaction among biological and environmental factors, and the genetic complexities of the crop. The advent of molecular and biochemical technologies, that began in the early 1990's and continues at an accelerated pace into the 21st century, undoubtedly will help to answer this genetic conundrum and increase efficiency of breeding for IDC-resistant crops.

3.1 *Glycine max* (L.) Merrill. – Soybean – Strategy I

The genus *Glycine* is divided into two subgenera: *Glycine* (perennials) and *Soja* (Moench) F.J. Herm. (annuals) (Hymowitz, 2004). The subgenus *Soja* includes the cultivated soybean *G. max,* a true domesticated species that would not exist in the absence of human intervention, and *G. soja* (L.) Sieb. and Zucc*.,* the wild annual soybean. Chromosome number of *G. max* is 2n = 40. It is an ancient polyploid that underwent an evolutionary process of allopolyploidization. All currently diploid species of the genus *Glycine* exhibit diploid-like meiosis, and are primarily inbreeders or self-pollinators that produce self seed. Crosses between *G. max* and *G. soja* are frequently fertile. This approach has been used in breeding to develop soybean cultivars for specialty types, such as small-seeded varieties.

IDC in soybeans has been recognized since the 1970's and significant improvements in IDC resistance have been achieved through long-term,

phenotypic evaluation and selection in field plantings on calcareous soils. The complex inheritance of IDC, which is population dependent (Cianzio and Fehr, 1980; Cianzio and Fehr, 1982; Weiss, 1943) and influenced by a significant genotype x environment interaction, poses however, important constraints to breeding efforts.

The soybean genome is of average size compared to many other plants (Shoemaker *et al.*, 2004) and it is comprised of about 1.1 Mbp/C (Arumuganathan and Earle, 1991). It is approximately seven and one-half times larger than the genome of *Arabidopsis*, and two and one-half times larger than rice. Still the soybean genome is less than half the size of the corn genome and more than 14 times smaller than the genome of bread wheat (*Triticum aestivum* L.).

It has been shown that homologous segments of soybean linkage groups (LGs) have a high degree of synteny with large portions of single chromosomes in *Phaseolus* and *Vigna* (Lee *et al.*, 2001). Each of the duplicated and homologous regions among the legumes is in turn homologous with duplicated regions of *Arabidopsis.* This retained synteny between *Arabidopsis* and soybean (Grant *et al.*, 2000; Lee *et al.*, 2001) could be of importance in attempting future genetic transformations of soybean.

A search of the Soybase Database (August 16, 2004) (www.soybase.ncgr.org) identified a total of 36 quantitative trait loci (QTL) associated with Fe. Molecular markers from six of the QTLs examined in a tester population, however, failed to identify Fe-efficient genotypes (Diers *et al.*, 1992), and therefore the role of these QTLs in Fe efficiency remains unconfirmed. The other 30 are distributed among eight different LGs: A1, B1, B2, G, H, I, L, and N. LGs B2 and N possess the largest number of Fe-associated QTLs. Percentage of the total variation explained by the QTLs ranges from 9.6 to 80%, suggesting the possibility that major genes for IDC resistance and Fe efficiency may be located in some of these chromosomal regions. The fact that so many QTLs have been associated with Fe further demonstrates the complicated inheritance of the trait in soybean (Cianzio, 1997). Heritability of each of the 30 QTLs was also calculated and ranged from 37.4 to 82.4. These values are an indication that selection for improved IDC resistance using some of these QTLs might be beneficial. These studies were all undertaken to identify molecular markers useful for marker-assisted selection (Diers *et al.*, 1992; Lin *et al.*, 2000a and 2000b). No distinctive function has been determined for any of the 36 QTLs mentioned previously. Their association with Fe efficiency, however, was confirmed in the research where plants were subjected to Fe deficiency either in field plantings on calcareous soils or in nutrient solution (Diers *et al.*, 1992; Lin *et al.*, 2000a and 2000b). Further functional characterization of these QTLs has not yet

been done and hindered by the lack of genetic information available for Strategy I genes in soybean.

In spite of the synteny between soybean and *Arabidopsis*, genes associated with any of the steps of Strategy I, either reductase or iron transporter, have not yet been identified in the soybean genome. A query under the category of 'Reaction or Pathway' in Soybase showed six reactions associated with Fe. Two pathways involving ferroleghemoglobin were identified in soybean, which are associated with nodules and the respiratory chain. The remaining reactions were not previously identified in soybean; however, the reactions were included on the basis of synteny between soybean and the specific species. Two reactions involved Fe reductases requiring NADH as a co-factor, however, these genes were identified in pea (Waters *et al.*, 2002). The two remaining reactions were respectively identified in *Arabidopsis* and potato.

Improvement of Fe efficiency in soybean has been achieved mainly through classical breeding approaches. In spite of advances in transformation and genetic engineering tools in soybean (Parrott and Clemente, 2004), genetic transformation to improve Fe efficiency in soybean had not been attempted until recently by T. Clemente (University of Nebraska; personal communication). In his ongoing work, Clemente introduced the gene *FRO2* from *Arabidopsis* into soybean. In a subset of the transformed soybean lines, evaluation of their Fe-efficiency is underway through examining chlorosis expression and Fe-chelate reductase activity in nutrient solution and field tests. The research is in progress in collaboration with M. Grusak (USDA, Childrens Nutritional Research Center, Houston, TX) and undoubtedly will be followed by other attempts in the near future.

The complex inheritance, environmental interaction, and the additional difficulties in screening and selecting for the most Fe-efficient genotypes are all reasons for considering genetic transformation as a possible tool to improve the Fe efficiency of soybean. An *a priori* advantage of this technique includes the introduction of genes with known function from phylogenetically-related plant species and model species. The disadvantages or yet unknown consequences of genetic engineering using orthologous genes might be the potential effect those genes may have on overall yield performance in the soybean plant. Work is in progress using microarray analyses, which will identify candidate genes in soybean for Fe efficiency and may help to circumvent any potential difficulties and unknowns using genes from non-related species in genetic transformation of soybean (O'Rourke and Shoemaker, Iowa State University; personal communication). Conversely, soybean genes identified for Fe storage in the seed have been used in genetic transformation of other plant species, as is the case of

soybean ferritin expressed in tobacco and other plant species (Jiang *et al.*, 2004).

3.2 *Oryza sativa* L. – Rice – Strategy II

The genus *Oryza* comprises 23 well-defined species (Chatterjee, 1948) of which only two are cultivated being *Oryza sativa* the major cultivated species. *O. sativa* is grouped into three varietal types: indica, predominantly distributed in the tropics and subtropics; japonica, grown only in temperate and subtropic regions; and javanicas, found mostly in Indonesia (Coffman and Herrera, 1980). Rice is a self-pollinated crop, with rare occurrences of natural cross-pollination, normally less than 1% (Beachell *et al.*, 1938). *O. sativa* has been crossed with other members of the series *Sativae*, with the resulting F_1's often sterile, as is the case for hybrids between japonica and indica groups (Jennings, 1966). Kuwada in 1910 determined chromosome number of cultivated rice as 2n = 24 (Fukui, 1996). Presence of six different genomes from A to F in the genus *Oryza* was determined, being the D genome only differentiated if in combination with the C genome in the amphiploid CCDD species (Fukui, 1996).

Since 1972, it has been recognized that chlorosis of young waterlogged rice plants was caused by the absence of Fe-solubilizing substances secreted by roots and diffused into water (Mori *et al.*, 1991). In 1984, research conducted using a classical genetic approach established that tolerance to IDC in semidwarf (PBN1) rice is controlled by a single, dominant gene Ic (Nerkar *et al.*, 1984). From the available information, research on Fe-related issues and progress to date has been made mainly in the biochemical and molecular aspects of Fe acquisition in rice.

The TIGR Rice Genome Database (www.tigr.org) showed 33 genes associated with Fe (August 10, 2004), distributed throughout 11 of the 12 rice chromosomes. Six genes were associated with Fe reduction. Three genes were associated with Fe transport, of which two are considered transporters for phytosiderophores with amino acid similarity to YS1 found in maize. In addition, six genes of the ZIP transporter family have also been identified in rice. The remaining genes associated with Fe were not identified as involved in Fe acquisition. In every case, the majority of the genes have been assigned to a particular chromosome. A new Fe-transporter gene has been recently identified and cloned from rice (Bughio *et al.*, 2002), however, the Fe transporter (*OsIRT1*) has not yet been included in the Rice Genome Database. *OsIRT1* is highly homologous to the metal transporter gene *IRT1* from *Arabidopsis* (Eide *et al.*, 1996). This gene may encode a functional iron transporter, since transcripts were predominantly expressed in the roots and were induced by Fe deficiency (Bughio *et al.*, 2002). These observations

indicate that rice possesses an iron uptake system for Fe^{2+}, in addition to a phytosyderophore-mediated Fe^{3+} transport system, suggesting a new mechanism of iron uptake that combines both Strategy I and Strategy II. A search of the database using the word 'nicotianamine' as query resulted in the listing of six genes.

The information collected directly from rice and summarized in the rice genome database has not been utilized to genetically improve Fe efficiency in the rice crop. However, elucidation of genes and metabolic pathways in PSs production and Fe transporters in other plant species opens up the possibility of using genetic engineering to improve Fe efficiency of rice. In fact, Fe-efficiency improvement in rice is mainly being accomplished by generation of transgenic plants, using as donors, species also possessing the Strategy II mechanism. For instance, it has been established that susceptibility to Fe deficiency in rice results from low amounts of secretion of deoxy-MA under Fe-deficient conditions and that secretion stops after seven days of Fe starvation (Mori *et al.*, 1991). Additionally, expression of genes involved in PS synthesis depends on plant growth stage (Mori *et al.*, 1991). Barley secretes much higher amounts and more kinds of PS over a longer period of time than does rice, which makes barley more resistant to IDC than rice (Mori *et al.*, 1988). Work to identify other chelators in rice plants has also been done by growing genotypes under Fe-sufficient conditions (Fushiya *et al.*, 1982), which has allowed isolation of NA from oats and rice, further indicating the importance of NA in cellular Fe transport and metabolism. Isolation work using roots of *Hordeum vulgare, Avena sativa* and *Secale cereale* allowed identification of MA, avenic acid, hydroxymugineic and 2-deoxymugineic acid (Fushiya *et al.*, 1982). The structural similarity among these compounds reveals their close biosynthetic relationships and the compatibility of gene transfers among species to produce additional PSs within a given species.

Transgenic rice has been engineered using genes from barley (Kobayashi *et al.*, 2001; Mori *et al.*, 2001). Rice plants were transformed with different genes of the PS biosynthetic pathway using the *Ids3* promoter from barley to control gene expression (Mori *et al.*, 2001). The results indicate that this is a possible avenue to improve Fe efficiency in rice, since several transgenic rice lines showed improved growth regarding plant height than the wild-type control. It was also determined that transformed rice with the *Ids3* gene secreted MA in addition to deoxyMA, which is the only PS produced by the wild type under Fe-deficient conditions.

Recent work indicates that genes cloned from other graminaceous species will serve to improve Fe efficiency in rice through genetic transformations. The cloning of two genes of the NAAT group (*naat-A* and *naat-B*) for nicotianamine aminotransferase (NAAT), a key enzyme involved in the

biosynthesis of MAs, are indications that new improvements in Fe efficiency could be expected (Takahashi *et al.*, 1999). The enzymes increase activity after the onset of Fe deficiency, which is correlated with the amount of MAs secreted. Several NA synthase genes also involved with NAAT have been recently cloned from barley (Higuchi *et al.*, 1999) and the work using these genes in genetic transformation is already underway in Mori's lab (Mori, 1999).

New candidate genes for genetic transformation are also being identified. Negishi *et al.* (2002) reported that approximately 200 clones were identified as Fe-deficiency inducible genes in a microarray that included cDNAs of rice EST clones to examine gene expression profiles in barley roots during Fe-deficiency stress. Of those, approximately 50 exhibited different transcription levels in Fe-deficient roots at two different times of the day, noon and night. A group of genes seems to be associated with this daily cycle, providing further evidence that genes are involved in the diurnal secretion of MAs. A recent report by Nishizawa (2004) indicates that 18 putative YS1-like genes were identified in the rice genome and named *OsYSL* (Nishizawa, 2004). Six of these genes were present in roots or leaves of Fe-sufficient or Fe-deficient rice plants (Koike *et al.*, 2004).

3.3 *Zea mays* – Maize – Strategy II

Zea mays L. is an extremely phenotypically variable grass species which in conjunction with seven other genera are included in the family *Maydeae* (Russel and Hallauer, 1980). Three genera are native to the Americas, *Zea, Euchlaena,* and *Tripsacum,* and five others are native to southeastern Asia and Australia. The maize plant is monoecious, with male flowers in the tassel and female flowers on the ear shoot, and the species is primarily cross-pollinated. Intergeneric crosses of *Zea* have been successful with *Euchlaena* (teosinte), *Tripsacum, Saccharum* (sugarcane) and *Coix,* although not all the seed is fertile. The maize genome is organized into 10 chromosomes with 2n = 20 and its genome is about 2.4 x 10^9 base-pairs in total (Brendel *et al.*, 2002).

Maize genotypes affected by Fe deficiency develop typical chlorosis symptoms of interveinal yellowing of leaves with veins that remain somewhat green, resulting in a striped appearance. It has long been recognized that there are differences in Fe efficiency among genotypes, with the ys_1/ys_1 (*yellow stripe1* mutant) being less efficient than WF9 (Brown and Ambler, 1970). Research conducted to characterize the *yellow stripe1* mutant identified it as being deficient in Fe^{3+}-PS uptake (Jolley and Brown, 1991), therefore YS1 had been suggested to be an Fe^{3+}-PS transporter (von Wirén *et al.*, 1994). Cloning of *ys1* and additional studies in yeast confirmed

the YS1 role in phytosiderophore uptake (Curie *et al.*, 2001; Roberts *et al.*, 2004).

A search of the TIGR Maize Database (www.tigr.org) identified 41 sequences of gene products associated with Fe (August 10, 2004). Different from the Rice Genome Database, the sequences have not yet been assigned to specific chromosomes. The sequencing of the gene products is complete for eight of the genes, while for the remaining 33 partial sequences have been determined. Homology between the *Zea mays* gene products with other organisms is also being established and listed in the database. There are 18 different plant and bacteria species with which maize has some, partial or complete homology. Among the graminaceous species, homologies in gene product sequencing have been established with *Oryza sativa,* and *Hordeum vulgare*, five genes in total. Homologies were also established with six gene sequences identified in *Citrus junos.* Two sequences were similar to *Arabidopsis thaliana*, and one each was similar to sequences in garden pea and potato. The other species with which homologies have been established are mainly with bacteria. A query for the term 'iron reductase' yielded no matches within the database, as well as no matches using the key word 'iron reduction'. There were three sequences associated with YS1. A more general query using the key word 'iron transporter' resulted in five sequences associated with the trait, being three of them common with the YS1 sequence. Five sequences were associated with transporters of the ZIP family. The remaining sequences associated with Fe pertain to other metabolic pathways. A search of the database querying for 'nicotianamine' resulted in the identification of six genes.

Improvement of IDC resistance in maize has been mainly done by using genotypes tolerant to Fe deficiency in commercial hybrids. From the available information, the data gathered in the Maize Database does not appear to have been used in breeding for improved genotypes. The work reported on *YS1* (Curie *et al.*, 2001), and microarray analysis of gene expression during Fe-deficiency (Negishi *et al.*, 2002) has been done to elucidate gene expression and function of genes associated with Fe transport, and does not appear to be part as of yet of a concerted effort to improve Fe efficiency in maize. However, the isolation of *YS1* may give promise to improve Fe acquisition in maize by manipulation of that gene. Curie *et al.* (2001) showed that steady-state levels of *YS1* mRNA were increased by Fe starvation in the maize roots. Another gene related to *YS1, YS3*, has been recently characterized by Römheld's group (Römheld *et al.*, 2004). Their results indicate that *ys3* might be defective in PS secretion, because upon Fe addition with Fe hydroxide in culture, the mutant *ys3* became green, while mutant *ys1* remains chlorotic.

Recent work by Brendel *et al.*, (2002) indicates that resources provided by the *Arabidopsis* genome cannot adequately substitute for more extensive maize genome sequencing. Genome organization is very different between the two species, and significant differences have also been detected in their proteomes, particularly with respect to traits of agronomic importance in maize, such as genes involved in plant-pathogen interactions, reproduction, and development and function of specific tissues. Synteny within the graminaceous species (Negishi *et al.*, 2002), however, and a more profound knowledge of the maize genome will provide the necessary tools to approach molecular breeding and improvement of Fe efficiency in maize.

3.4 Comparison among genomic databases of soybean, rice and maize

Charlson and Shoemaker (unpublished data) compared Fe strategies and genes identified in several plant species using EST databases, among them soybean, rice and maize. They have determined that the three species possess genes with amino acid similarity to Strategy I components, namely Fe reductase, and Fe^{2+} transporters. In addition, *FRD3*-like ESTs were identified in all three species. The species also share some homologous genes involved in Strategy II, namely NA synthase and *YS1*. However, the soybean EST database lacked the presence of genes with homology to *NAAT* and *IDS3* found in maize and rice EST databases. Although the homologous genes share similar amino-acid sequences, their actual gene function may vary in the respective plant species. To date, there is no available information of studies in nutritional genomics among species that could elucidate this concern.

4. FUTURE OUTLOOK

Genomic information in each of the three species related to important vital pathways is gathered on an every day basis, increasing biochemical and physiological understanding of processes in living organisms. Nevertheless, a comparison among the three species shows a great deal of variation at least in information related to Fe uptake. Of the three, rice genomic research appears to have a more defined goal, and it looks closer to reaching possible solutions. Genetic transformations in rice are already under way in laboratory conditions with very successful results (Guerinot, 2001; Mori, 1999). Final confirmation of how feasible and applicable this work is to rice in commercial production will require extensive field evaluation. Only then, yield effects of improved genotypes will be measured, and final steps may

be taken for use in commercial plantings. In maize, genomics of Fe efficiency does not seem to have reached yet a similar level of progress as in rice. However, as maize is a member of the graminaceous family, it will benefit from progress achieved in other grasses, such as rice. In soybean, work conducted in Fe efficiency has almost exclusively concentrated on the identification of QTLs. Of the three species, it appears that the least amount of progress in the genomic aspects of Fe uptake has been made for soybeans. Progress in the three species is expected since genomic knowledge in plants is continuously expanding.

Complexity of Fe uptake process suggests that genetic improvement in crops may be achieved by the combination of traditional breeding and genetic engineering techniques. As more is known about gene function across plant species and development of more efficient tools for plant transformation, techniques for plant engineering may be routinely used to improve Fe acquisition in plants. Similarities in genomes of higher plants suggest that genes associated with Fe have been conserved and are present in a wide range of organisms. Undoubtedly, genetic improvement of Fe efficiency in plants will be accomplished in very creative ways, with the help of genome information, breeding, and genetic transformation and engineering.

Some thoughts and words of caution may be however necessary. Conventional plant breeding has been responsible for the very significant increases in the genetic yield potential of crop plants and the increase in abiotic- and biotic-stress resistance (Cassells and Doyle, 2003). This has been accomplished with support from agronomists, plant physiologists, plant pathologists, and geneticists. However, if genes need to be introduced from reproductively incompatible sources, only genetic engineering has the capability to do it. The procedure will require field trials, just as in traditional breeding, which are also mandatory in transgenic crops prior to commercial and consumer acceptance. Conventional plant breeding therefore will continue to be important *per se,* and in the evaluation of interactions among newly introduced genes and genes present in the species under consideration.

In the particular case of Fe uptake, genetic engineering will face an additional challenge, which will affect traditional breeding strategies. Due to the complex biochemical nature of the Fe-uptake processes, multiple genes may be required for insertion through a single or very few transformation events. This will invoke another more complicated problem faced by the genetic engineering of today, i.e. the regulation of transgene expression, or genome position effect, not with just one gene, but with multiple genes involved in different stages of the Fe pathway. In breeding this will translate in the conduct of extensive field trials and the evaluation of complex

genomic interactions, before the transformed crop is ready for commercial production. The future looks promising and challenging as new questions arise in the search and solution of existing problems.

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