Chemical Strategies for Iron Acquisition in Plants

Dorothee Staiger*

Abstract: Iron is an essential element for plant nutrition. Although iron is the fourth most abundant element (3%) of the earth's crust, it is not readily available because of its low solubility. Therefore, plants need an active mechanism to extract iron from the soil. They have evolved several chemical strategies to acquire iron ions and the physiology of these mechanisms has been known for a long time. Only recently, the use of molecular genetic approaches has led to a biochemical and molecular characterization of the players involved, thus providing an entry to the manipulation of iron uptake in plants.

Introduction

1.1. The Role of Iron in Plant Metabolism

In 1882 the plant physiologist Julius von Sachs became aware of the importance of iron for plant nutrition. He investigated the consequences of submerging roots into an iron-free medium by growing plants in hydroponic cultures. He observed that newly formed leaves remained white because they were not able to produce the green pigment, chlorophyll, and concluded that this disease, chlorosis, is the result of iron deficiency.[1] This experiment demonstrated that iron is essential for chlorophyll biosynthesis.

The importance of iron for metabolism is founded upon its ability to form two stable ions, Fe^{II} and Fe^{III}. Accordingly, iron ions are involved in most redox processes in the electron transport chains of photosynthesis and respiration which serve to transform energy from electron transport into ATP, the energy source of the cell. Iron is also important for symbiotic nitrogen fixation in root nodules of legumes. Iron is contained in the subunits of the nitrogenase enzyme that reduces N_2 to NH_3, as well as in leghaemoglobin that binds molecular oxygen in the root nodules.

[*] Dr. D. Staiger
Institute for Plant Sciences
Swiss Federal Institute for Technology
8092 Zurich (Switzerland)
Fax: (+41) 1-632-1081
E-mail: dorothee.staiger@ipw.biol.ethz.ch

1.2. Threats of Excess Iron

The property of iron ions to catalyze one-electron transitions requires a limitation of the cellular iron concentration since iron promotes the formation of toxic oxygen species. Superoxide anions are formed as intermediates during the reduction of molecular oxygen to H_2O within the cell. These anions reduce Fe^{III} to Fe^{II} (Scheme 1), which in turn catalyze

Reduction of Fe^{III} by superoxide anions

$$\cdot O_2^- + Fe^{III} \longrightarrow O_2 + Fe^{II}$$

Fenton reaction

$$Fe^{II} + H_2O_2 \longrightarrow Fe^{III} + OH^- + \cdot OH$$

Sum: Haber–Weiss reaction

$$\cdot O_2^- + H_2O_2 \longrightarrow \cdot OH + OH^- + O_2$$

Scheme 1. Toxicity of iron ions within the cell. The Haber–Weiss reaction is the sum of the reduction of Fe^{III} ions by superoxide anions and the Fenton reaction, the Fe^{II}-catalyzed degradation of H_2O_2 to highly reactive hydroxyl radicals.

the decomposition of H_2O_2 to highly reactive hydroxyl radicals (the Fenton reaction), which damage cellular components such as DNA and lipids. Such an excess of reactive oxygen species is designated oxidative stress.[2, 3] In pea mutants that accumulate excess iron, for example, cell death even occurs, which leads to so-called necrotic lesions in leaves. This situation is analogous to a hereditary disease in humans in which enhanced iron uptake correlates with highly enhanced incidence of liver cancer.

How do plants cope with excess iron? On the one hand, as a preventive means, an antioxidative system destroys superoxide radicals and H_2O_2 before they come into contact with iron. Ironically, iron and heme groups are essential cofactors of the peroxidase and catalase enzymes which decompose H_2O_2. On the other hand, excess iron is stored in a multimeric protein, ferritin, that can accomodate up to 4500 iron ions.[2]

1.3. Iron Uptake

Control of iron uptake represents another approach the plant can adopt to balance its iron content. Although iron

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ranks fourth (3%) among the elements in the earth's crust, it is not readily available for plant nutrition. Iron primarily occurs in the form of insoluble oxyhydroxide polymers, such as goethite (α-FeOOH) and hematite (α-Fe_2O_3), which are generated by weathering. Free iron ions occur at neutral pH values only at a concentration of about 10^{-17} M; ferrous ions that are more soluble are readily oxidized to ferric ions, which again precipitate.[4] The low solubility of ferric ions therefore does not guarantee a sufficient supply of iron for the roots. Plants therefore require an active mechanism to release iron from Fe^{III} oxide hydrates and to absorb it from the soil.

Iron excess is found mainly on waterlogged soil with anaerobic conditions, such as rice fields. Here, Fe^{III} ions are readily reduced to more soluble Fe^{II} ions. The excess accumulation of Fe^{II} ions in the rice plants results in the well-known bronzing phenomenon, caused by oxidative stress.[2, 3]

Plants have evolved three chemical strategies to acquire iron: release of protons from the root surface to enhance dissociation of Fe^{III} oxides, reduction, and chelation.[3, 5] These mechanisms have been well described at the physiological level and several mutants defective in iron uptake have been identified during the last few decades. However, the use of molecular genetic approaches has only recently led to the biochemical and molecular characterization of the players involved.

2. Strategy I: Reduction

Most plants, except the grasses, activate a battery of mechanisms—the so-called strategy I, which should be distinguished from strategy II of the grasses which will be discussed subsequently.[5]

2.1. Elements of Strategy I

Strategy I plants excrete protons from the root surface to lower the pH value in the immediate vicinity of the root, the so-called rhizosphere. It is likely that this is accomplished by activation of a proton-pumping ATPase, an enzyme that transfers protons through the cell membrane to the outside at the expense of ATP. Acidification of the soil shifts the equilibrium towards dissociation of $[Fe(OH)_3]$ complexes: lowering the pH value by one unit increases the solubility of Fe^{III} ions by a factor of a thousand.[6]

Furthermore, the capacity to reduce ferric ions in an NADH-dependent manner increases at the root surface. Ferrous ions are about 10^{16} times more soluble than ferric ions at neutral pH. Ferrous ions are the substrate for a specific uptake system.[7] In addition, plants increase the surface available for iron uptake by an increased formation of root hairs (appendices of the outermost cell layer). The elements of strategy I are shown in Figure 1. A sensor that measures iron concentration within the plant has remained elusive.

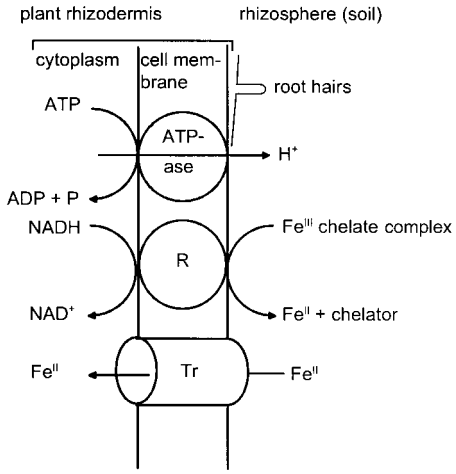

Figure 1. Elements of strategy I (adapted from ref. [5]). The outermost cell layer of the root, the rhizodermis, and the immediate vicinity of the soil, the rhizosphere, are depicted. All plants, except the grasses, respond to iron limitation by acidifying the rhizosphere, increased reduction of an Fe^{III} chelate by an NADH-dependent reductase (R) at the root surface, and Fe^{II} uptake by a specific, inducible uptake systeme (Tr). Moreover, the root surface is increased through the formation of additional root hairs.

2.2. Identification of Fe^{III}–Chelate Reductase through a Genetic Approach in the Model Plant *Arabidopsis thaliana*

A genetic approach in the model plant *Arabidopsis thaliana* (Figure 2) has led to the identification of components of the iron-uptake system. *Arabidopsis* is a weed of the cruciferous plant family. It has developed into the model system of plant molecular biologists because of its short generation time of a few weeks and its small genome that has been entirely sequenced.[8]

Figure 2. *Arabidopsis thaliana* is a small member of the cruciferous plants. It has developed into the model plant of plant molecular biologists because of its short generation time of a few weeks and its small genome, whose entire sequence is known.[8] The bar corresponds to 5 cm.

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Arabidopsis is a strategy I plant and relies on solubilization of iron by an increased NADH-dependent Fe^{III}-reducing capacity at the root surface. A genetic screen for mutants that do not enhance this Fe^{III}-reducing capacity in times of iron deficiency has been initiated to identify the reducing activity molecularly.[9] Towards this end, *Arabidopsis* seeds have been treated with ethyl methane sulfonate (EMS), a mutagen that modifies bases of the genome. In the case where such a mutation affects an essential amino acid of a particular protein, a defect is observed in the plant. The offspring of the mutagenized population was screened for individuals with a defect in their iron-reducing capacity by a colorimetric test for the product of the reduction: ferrous ions form a complex with the ferrozine dye whose appearance can be followed photometrically at 562 nm.

In this way, three mutants have been identified, *frd1-1*, *frd1-2* and *frd1-3* (frd = ferric reductase deficient). These mutants do not show an increased Fe^{III} reductase activity upon iron depletion, in contrast to wild-type plants.[9] Genetic tests revealed that the three mutations affected the same gene locus.

The identification of the defective gene in these mutants took advantage of a concomitant approach to clone plant Fe^{III}–chelate reductase by homology to the known yeast Fe^{III}–chelate reductase. Towards this end, chemically synthesized oligonucleotides corresponding to conserved regions of the yeast gene were used as starting molecules for enzymatic amplification from the *Arabidopsis* genome by the polymerase chain reaction.[10]

Indeed, part of an *Arabidopsis* gene was amplified that codes for a putative reductase. The predicted protein shows, among others, homology to human phagocyte NADPH oxidase. This enzyme catalyzes the reduction of O_2 to O_2^- by transferring one electron across the cell membrane onto O_2. The newly identified *Arabidopsis* protein features hydrophobic regions indicative of localization within a membrane (Figure 3). On the cytosolic sides there are binding sites for the electron donor NADH and the cofactor FAD. The membrane-localized part of the molecule contains binding sites for the heme groups that mediate electron transport to the outside.[10]

Is this protein indeed connected to the defect in the *frd* mutants? The transformation of the wild-type gene for the

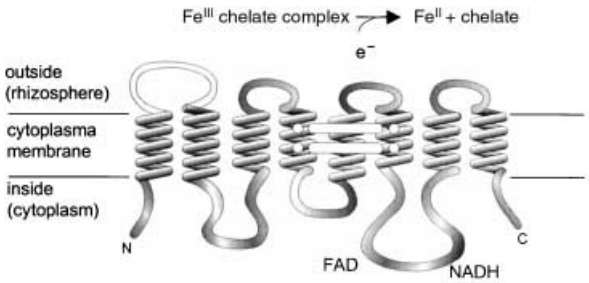

Figure 3. Schematic structure of the Fe^{III}–chelate reductase (adapted from ref. [10]). The reductase has hydrophobic regions indicative of membrane-spanning domains. On the cytosolic site there are binding sites for the electron donor NADH and the cofactor FAD. In the part of the protein that is localized within the membrane there are two histidine residues (open circles) which serve as attachment sites for the heme groups that mediate the electron transport.

Fe^{III} reductase into the *frd1* mutants results in their ability to reduce Fe^{III} ions being restored. Furthermore, point mutations were found in the reductase genes of the mutants that led to defective proteins, which indicated that the *frd* mutations indeed affect the reductase-reducing Fe^{III} centers at the root surface.[10]

2.3. Identification of Components of the Iron-Uptake System by Functional Complementation of Yeast Mutants

How do Fe^{II} ions formed by this reduction step finally get into the cell? Functional complementation in yeast was applied to identify iron transport proteins (Figure 4). This

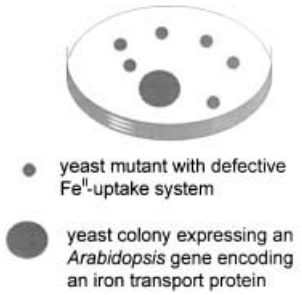

Figure 4. Identification of an *Arabidopsis thaliana* iron-transport protein by functional complementation in yeast. A yeast mutant with a defect in two Fe^{II}-uptake systems grows only poorly on a medium with a low iron concentration and forms small colonies. A number of *Arabidopsis* genes was introduced into this mutant. Colonies that subsequently grow better express an *Arabidopsis* protein mediating Fe^{II} uptake.[11]

technique relied on a yeast mutant defective in two iron-uptake systems. It grows only poorly on substrates with reduced iron content, as indicated by the formation of small colonies. A collection of *Arabidopsis* genes was introduced into this mutant. Colonies that grow better and thus have a larger diameter should express an *Arabidopsis* protein mediating Fe^{II} uptake.

In this way the *Arabidopsis* protein IRT1 (IRT = iron-regulated transporter) encoding a putative iron-transport protein was isolated.[11] The derived protein is predicted to have eight membrane-spanning domains. No homology was found to known iron-transport proteins from yeast or the bacterium *Escherichia coli*, which indicates that IRT1 is the prototype of a new class of transport proteins.

The transport properties of IRT1 were tested after heterologous expression in yeast. The measurement of the uptake of radioactive iron ions in the presence and absence of the reductant ascorbate revealed that Fe^{II} ions are the preferred substrate. This observation is consistent with a physiological role for IRT1 in the uptake of Fe^{II} ions, the product of NADH-dependent reduction.[11] It is frequently observed that not only iron, but other toxic ions also accumulate in the plant in response to iron deficiency. To test whether IRT1 can transport other ions several transition metals were tested for their ability to compete with uptake of radioactive iron ions.[11] A 10-fold excess of Cd^{II} ions inhibits Fe^{II} uptake, whereas Mn^{II} and Zn^{II} ions are inhibitory only at a 100-fold excess.

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These observations suggest that IRT1 may have a broad substrate specificity and may transport toxic ions such as cadmium into the plant.

The importance of single amino acids for the transport process was investigated by site-specific mutagenesis.[12] Replacement of three histidine residues and a glutamic acid residue by alanine eliminates transport activity, thus indicating that these residues may bind the metal ions during the transport process. Mutation of glutamic acid 103 eliminates the ability to transport zinc, but does not affect the transport of iron, manganese, and zinc ions. Mutation of aspartic acid 100 eliminates the transport of iron and manganese ions. Mutation of aspartic acid 136 also eliminates transport of iron and manganese, and impairs uptake of cadmium ions, thus leaving only zinc ions as a substrate. Thus, single amino acids were indeed determined that convey substrate specificity to the transporter.

This finding represents a first step towards a targeted modification of the transport profile of a protein mediating metal uptake in plants. Such a modified protein ultimately may prove useful to manipulate a plant's metal content by selective uptake of specific cations, such as iron, but elimination of others, such as the toxic cadmium ion.

Recently, a second iron transport protein closely related to IRT1, namely IRT2, was identified in *Arabidopsis*.[13] Its expression in the yeast mutant led to the uptake of iron and zinc, but not manganese and cadmium. The expression of the *IRT2* gene in the outermost cell layers of the root and in the root hairs suggests that IRT2 may mediate iron uptake from the soil.[13]

3. Strategy II: Chelation

Grasses, for example, barley and maize, follow strategy II. It is based on chelation, and takes advantage of iron's propensity to form complexes through coordinative bonds. When iron deficiency occurs, the plants synthesize and secrete hexadentate chelators that bind Fe^{III} ions from complexes in the soil and thus keep it in solution.[3, 5] These chelators are designated "phytosiderophores", in analogy to iron-uptake systems of microorganisms that secrete iron chelators designated as "siderophores", from the Greek "iron bearers".[14] The complexes of iron with the phytosiderophores are reimported into the root through specialized transport systems (Figure 5).[15]

The amount of phytosiderophores released into the soil correlates with the plant's tolerance of iron deficiency. Barley is relatively resistant against iron deficiecy, whereas rice plants that secrete only a few phytosiderophores are sensitive to iron deficiency (Figure 6).[5, 16]

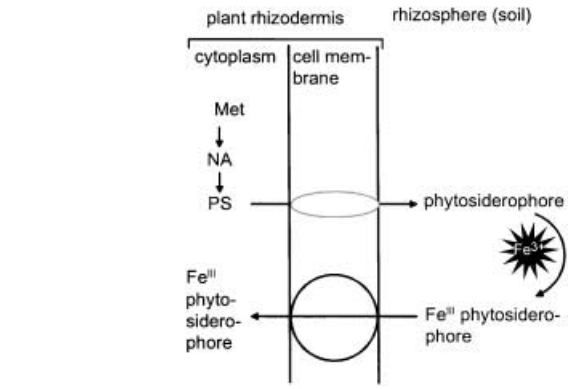


Figure 5. Elements of strategy II (adapted from ref. [5]). The outermost cell layer of the root, the rhizodermis, and the immediate vicinity of the soil, the rhizosphere, are depicted. Grasses respond to iron limitation by synthesis of phytosiderophores (PS) from L-methionine via the non-protein amino acid nicotianamine (NA). The phytosiderophores are released into the rhizosphere and bind Fe^{III} ions. The entire Fe^{III}–phytosiderophore complexes are taken up into the cells of the root surface through a specific, inducible transport system.

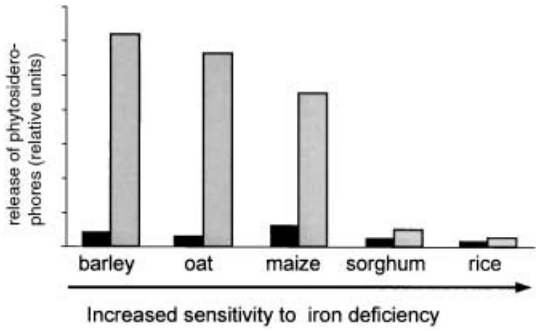
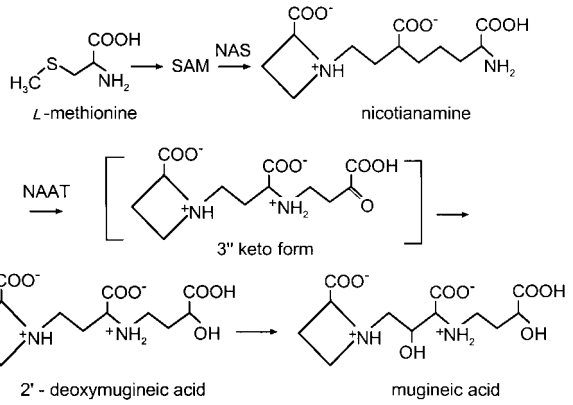


Figure 6. Correlation of the release of phytosiderophores and resistance to iron deficiency (adapted from ref. [5]). The amount of phytosiderophores released into the soil correlates with the plant's tolerance of iron deficiency. Barley is relatively resistant to iron deficiency whereas rice that secrets only a small amount of phytosiderophores is very sensitive to iron deficiency. Black bar: preculture in the presence of iron; gray bar: preculture in the absence of iron.



Scheme 2. Biosynthesis of phytosiderophores (refs. [17–19]). SAM = (*S*)-adenosylmethionine; NAS = nicotianamine synthase; NAAT = nicotianamine aminotransferase.

3.1. Biosynthesis of Phytosiderophores

Phytosiderophores are produced according to the iron demand in the cell. Under iron limitation, the corresponding biosynthetic enzymes are induced (Scheme 2). L-methionine serves as a precursor molecule that is activated at the expense of ATP to (*S*)-adenosylmethionine.[17, 18] Polymerization of three molecules of (*S*)-adenosylmethionine and formation of an azetidine ring yields the non-protein amino acid nicotianamine. The enzyme nicotianamine synthase was purified from

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barley and cloned. It is a novel protein.[19] Nicotianamine is converted into the keto form through a transamination catalyzed by nicotianamine aminotransferase and subsequently reduced to 2′-deoxymugineic acid.[20] The six functional groups serve to bind the Fe^{III} ions as chelates. The introduction of additional hydroxy groups into 2′-deoxymugineic acid yields species-specific derivates of mugineic acid that are collectively designated phytosiderophores.[21] The additional hydroxy groups can increase the stability of the Fe^{III}–chelate complexes.[22]

Interestingly, nicotianamine was found not only in strategy II plants but also in all the plants examined. A mutant with iron-deficiency symptoms has been found in tomato. This mutant is unable to synthesize nicotianamine. Nicotianamine is thought to play a role in the transport of iron ions in strategy I plants such as tomato. It was shown that the mutant has a defect in nicotianamine synthase.[23] The products of the subsequent reactions are found only in grasses. The transfer of the amino group by nicotianamine aminotransferase is thus the key reaction that distinguishes grasses from the remainder of the plants.[24]

3.2. Identification of the Uptake System in Fe^{III}–Phytosiderophore Chelate Complexes

Whereas Fe^{III} ions are reduced to the more soluble Fe^{II} form and taken up by transport proteins such as IRT1 in strategy I plants, strategy II plants take up Fe^{III} ions complexed with phytosiderophores.[5] Recently, the special uptake system for phytosiderophores was identified by taking advantage of the maize *yellow stripe* mutant that cannot take up Fe^{III} phytosiderophores.[25] Thus, it suffers from iron deficiency and impaired chlorophyll biosynthesis, which results in the characteristic yellow stripes on the leaves. The mutation is caused by insertion of the transposon Ac (activator) into a gene essential for iron uptake. Transposons are DNA sequences that can change their position in the genome. By using this genetic element as a tool, the affected gene was isolated. It codes for a protein with 12 transmembrane domains. Its role in iron transport was investigated in the above-mentioned yeast mutant that is deficient in iron uptake and thus grows poorly under iron limitation. Upon expression of the maize *YS1* gene in the yeast mutant, the mutant is able to form colonies on medium containing Fe^{II}-deoxymugineic acid, which suggests that *YS1* indeed encodes a transporter for Fe^{III} siderophores.[25]

4. Summary and Perspecitve

Under iron limitation, strategy II plants, such as the grasses, rely on the production of phytosiderophores, hexadentate ligands, that are able to form complexes with Fe^{III} ions. The Fe^{III}–phytosiderophore complexes are subsequently taken up by the plants with the help of highly specific root-transport systems.[15] All other plants release protons into the soil to solubilize Fe^{III} ions, which are the substrate of an inducible Fe^{III}–chelate reductase. The product Fe^{II} ion is taken up into root cells through a specific metal transport protein such as *Arabidopsis* IRT1.[11] Strategy I is severely impaired on calcareous soils because of the high pH values and the low solubility of Fe^{III} ions. In contrast, phytosiderophores are capable of chelating iron even at higher pH values. This offers an ecological advantage to grasses on calcareous soils relative to strategy I plants.

What are the implications of an increased understanding of iron-uptake processes in plants for human nutrition? Iron deficiency is the most prominent of nutritional disorders.[26] As plants are the main iron source for the majority of the world's population, enhancing the iron content of crop plants could contribute to alleviate iron deficiency.

A prerequisite to manipulate the iron content is to understand the fate of iron in the entire plant: further issues include details about the translocation of iron within the plant, the distribution of iron to the organelles within the cell as well as regulation of the genes whose products are involved in uptake, distribution, and storage of iron.

To increase the iron content in a useful form for human nutrition iron has to be targeted to those plant organs that provide nutrition. Moreover, an increase in iron uptake by enhanced expression of transport proteins in transgenic plants requires that the simultaneous uptake of unwanted ions such as toxic Cd^{II} is avoided. Another process that relies on the uptake of toxic metal ions is known as phytoremediation, the decontamination of soil and water polluted with heavy metals with the aid of metal-accumulating plants.[26] In this case, plants should not transport the metal ions from the roots to the shoot in order to keep the biomass for disposal small. Progress in understanding iron uptake obtained with the model plant *Arabidopsis thaliana* provides an entry to these applications.

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