Chapter 5
Micronutrient Use Efficiency – Cell Biology of Iron and Its Metabolic Interactions in Plants

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Abstract Iron (Fe) is an intriguing nutrient due to its dual nature. Its redox properties make it essential for different vital processes in plant cells. But an excess of Fe can be toxic as it catalyses the formation of reactive oxygen species. Therefore Fe homeostasis must be tightly regulated. Different mechanisms contribute to the regulation, including the control of uptake, the intracellular chelation by different molecules and the partitioning into the organelles and storage locations. Despite its high abundance in soil, Fe solubility is extremely low. Fe availability represents a significant constraint to plant growth and plants have developed distinct strategies to ensure Fe solubilisation and uptake. The Fe-S clusters in the electron transport chain of mitochondria and chloroplasts represent an important sink of Fe. Recent observations suggest that a co-regulation exists between Fe and sulfur metabolism. This is most likely the outcome of the high demand for Fe and S required for the biosynthesis of Fe-S clusters. In the following chapter the uptake strategies and their regulation mechanisms will be introduced. Moreover, different aspects of the regulation of Fe homeostasis in the cell will be presented, including the partitioning in the organelles. In the last section different evidences towards the interaction between Fe and S metabolism will be discussed.

Keywords Iron • Micronutrients • Homeostasis • Regulation • Iron transporters • Deficiency • Partitioning

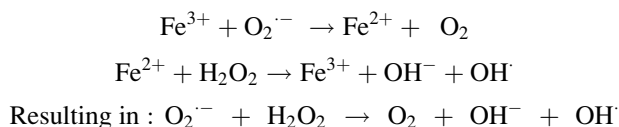## Introduction: Fe Importance for Plant Nutrition

Iron (Fe) is a chemical element with atomic number 26, which belongs, together with manganese, cobalt, nickel, copper and zinc, to the metals of the first transition series in the periodic table. Fe is the most abundant element found on planet Earth, as it constitutes a significant part of the inner and outer core. It is the fourth most

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common element in the crust, after oxygen, silicon and aluminium. As a transition metal, Fe can easily accept and donate electrons. Thus its oxidation state can vary in a broad range between -2 and $+6$, but the most common under current atmospheric conditions are $+2$ (ferrous iron) and $+3$ (ferric iron). This redox property of Fe and its capability to form complexes with different ligands make this element indispensable for different biological processes in all the living organisms. Indeed several proteins (Fe-proteins) found in different organisms rely on Fe as a cofactor for proper functioning. Fe is also essential for plant metabolism, where it participates in vital cellular functions such as photosynthesis, respiration and chlorophyll biosynthesis.

Despite its essential role for life, an excess of free Fe can be detrimental to the cell because it can react with oxygen catalysing the formation of reactive oxygen species (ROS) such as superoxide ($O_2^{\cdot-}$) and hydroxyl radical ($OH^{\cdot}$) via the Fenton reaction (reviewed by Hell and Stephan 2003):

$$Fe^{3+} + O_2^{\cdot-} \rightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\cdot}$$

$$\text{Resulting in}: O_2^{\cdot-} + H_2O_2 \rightarrow O_2 + OH^- + OH^{\cdot}$$

These radicals constitute a severe danger for the cell particularly the hydroxyl radical, which is very reactive and can indiscriminately oxidise DNA, polyunsaturated fatty acids in lipids (lipid peroxidation), amino acids in proteins and sugars.

To avoid potentially toxic reactions, protein-bound Fe is found incorporated into structures such as heme or coordinated with sulfur (S) to form Fe-S cluster. Heme contains a Fe atom in the centre of a large heterocyclic organic ring, the porphyrin, made of four pyrrolic groups joined by methine bridges. Among heme proteins, a fundamental role is played by hemoglobin and myoglobin in vertebrates as they contain a Fe atom which binds to oxygen. The most common heme proteins in plants are cytochromes, which participate in the electron transport process of mitochondria and chloroplasts. Other heme proteins are catalase and peroxidases that are involved in scavenging of ROS.

Fe-S clusters are versatile and ubiquitous cofactors of many different enzymes that participate in respiration, photosynthesis, DNA repair and replication, sulfur and nitrogen assimilation and ribosome biosynthesis (Balk and Pilon 2011). They are formed from Fe atoms and sulfur in the form of acid-labile sulfide and are bound to proteins via the sulfhydryl groups of cysteine residues. Different forms are found in plants, the most common are the 2Fe-2S and 4Fe-4S bound to four cysteine (Cys) residues. Other types include the 2Fe-2S Rieske-type cluster coordinated by 2 Cys and 2 His residues and 3Fe-4S liganded by 3 Cys (reviewed by Couturier et al. 2013).

Toxicity of free Fe ions may be avoided by chelation by different compounds such as the non-proteinogenic amino acid nicotianamine, citrate and the storage via ferritin proteins.

Ferrous Fe is relatively soluble but it is easily oxidised to ferric Fe by atmospheric oxygen. The solubility of Fe^{3+} is highly influenced by the soil pH. Indeed in alkaline conditions Fe^{3+} hydrolyses water producing $Fe(OH)_3$ that polymerises and precipitates together with inorganic anions. Free Fe^{3+} is soluble up to 10^{-6} M at pH 3.3, but in aerated soil at neutral-basic pH, the concentration of free Fe^{3+} and Fe^{2+} is estimated to be less than 10^{-15} M (Marschner 1995). This is much lower than the optimal concentration needed by plants that require between 10^{-4} and 10^{-8} M Fe^{3+}. This solubility problem strongly impacts Fe availability that represents a severe constraint for plant development and yield. In particular, Fe is considered the third most limiting plant nutrient after nitrogen and phosphorus. Hence, even though Fe is quite abundant in soil, Fe deficiency represents a major problem for worldwide agriculture in calcareous-alkaline soil, which comprises 30 % of all arable land. Upon Fe deficiency, plants display typical symptoms such as leaf chlorosis and reduced growth. Therefore the quest for Fe use efficient crop plants is a goal of plant breeding and biotechnology.

Plants are always dealing with the dual nature of Fe and have developed sophisticated mechanisms to ensure adequate Fe acquisition from the soil and at the same time to make it available for biological processes in the cell avoiding toxic reactions.

Fe content in crop plants, which constitute a widely utilised staple food, significantly influences Fe assimilation by the human population. Fe deficiency is one of the most diffuse nutritional problems in the world, with around 30 % of the world population affected according to World Health Organization (http://www.who.int/nutrition/topics/ida/en/). Therefore, understanding the mechanisms used by plants to cope with changing Fe availability is a prerequisite not only for improving crop yield but also for a positive impact on human nutrition.

In this chapter, the strategies adopted by plants for Fe uptake will be reviewed, with focus on mechanisms of regulation. The partitioning of Fe in the cell and the interaction with other nutrients such as sulfur will also be presented.

Fe Deficiency and Plant Responses

Higher plants use distinct strategies to ensure Fe solubilisation and uptake. In the 1980s, Römheld and Marschner (1986) divided plants into two groups according to their Fe uptake mechanisms. Dicotyledonous and non-graminaceous monocotyledonous plants belong to the Strategy I or reduction strategy group, whereas Poaceae to the Strategy II or chelation strategy group.

Strategy I is based on (1) soil acidification to increase Fe solubility, (2) reduction of Fe^{3+} to Fe^{2+} in the rhizosphere and (3) uptake of Fe^{2+} across the root plasma membrane (see Fig. 5.1). This strategy was first characterised in *Lycopersicum esculentum* (tomato) and *Pisum sativum* (pea) as model crop plants. Recently, most of the studies in this respect have focused on *Arabidopsis thaliana*, which represents a powerful tool for cell biology investigations. The genes responsible for the
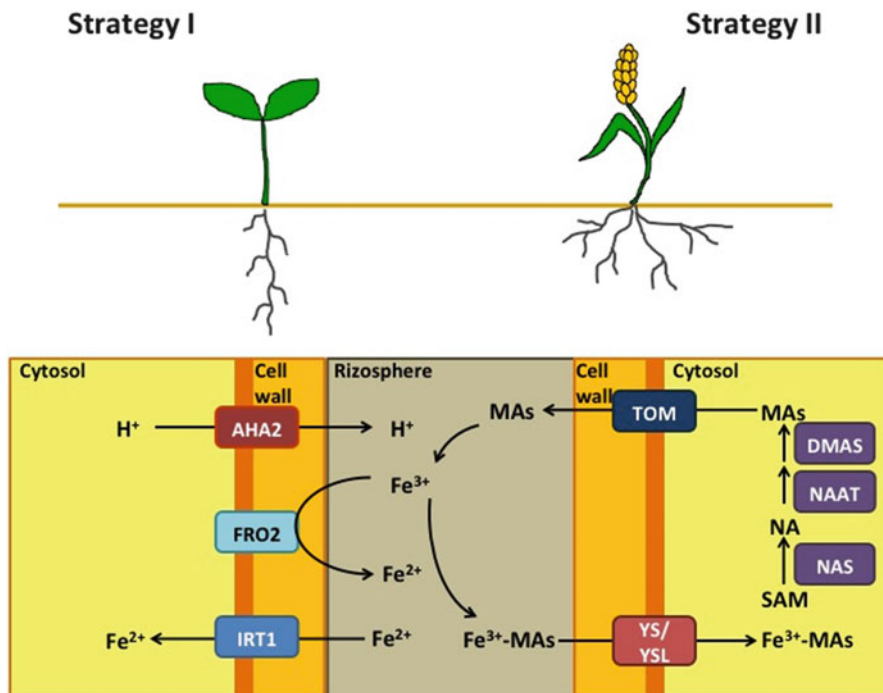
Fig. 5.1 Fe deficiency responses in plants. Strategy I (non-graminaceous plants) and Strategy II (graminaceous plants) are presented. In the *rectangles* the key enzymes of the two strategies are shown. Abbreviations: *AHA2 Arabidopsis* H$^+$-ATPase, *DMAS* deoxymugineic acid synthase, *FRO2* ferric chelate reductase 2, *IRT1* iron regulated transporter 1, *MAs* mugineic acids, *NAAT* nicotianamine aminotransferase, *NAS* nicotianamine synthase, *TOM* transporter of mugineic acids, *YS* yellow stripe, *YSL* yellow stripe like. YSL refers to orthologs of YS in plants other than maize

different steps of the strategy I have been identified and cloned and Fe deficiency results in an up-regulation of their expression. Firstly, the H$^+$-ATPase family (HA) excretes protons into the rhizosphere to increase Fe solubility (Palmgren 2001). In *Arabidopsis* the *HA2* gene particularly is induced in Fe deficiency (Santi and Schmidt 2009). The reduction of Fe^{3+} is catalysed by the ferric-chelate reductase oxidase 2 (FRO2) in *Arabidopsis* (Robinson et al. 1999) and by FRO1 in pea (Waters et al. 2002). FRO proteins are integral membrane proteins that belong to a superfamily of flavocytochromes and can transfer electrons from cytosolic NADPH to FAD across the plasma membrane (Robinson et al. 1999). *FRO2* was isolated as allelic to the *frd1* mutants in *Arabidopsis* (Yi and Guerinot 1996). These mutants are not able to induce the Fe chelate reductase activity, although they are still able to acidify the rhizosphere upon Fe deficiency. Moreover, these mutants cannot translocate radiolabeled Fe from root to the shoot when Fe is provided as chelated Fe^{3+}. Altogether these results shown that FRO activity is uncoupled from the HA activity and that Fe^{3+} reduction to Fe^{2+} is a prerequisite for the transport.

After reduction, the uptake of Fe^{2+} in the root epidermal cells is performed by the iron-regulated transporter 1 (IRT1) in *Arabidopsis* (Vert et al. 2003). Orthologs of IRT1 were cloned in both pea and tomato (Cohen et al. 1998; Eckhardt et al. 2001). The *Arabidopsis* knock-out mutant *irt1* is lethal unless plants are watered with an excess of Fe (Vert et al. 2002). Another transporter, IRT2, is also induced in roots when exposed to Fe shortage (Vert et al. 2001) but its knock-out mutant is not affected under normal Fe conditions. The attempt to complement the *irt1* mutant with *IRT2* driven by a constitutive promoter did not rescue the phenotype, showing that the two transporters have different roles in Fe uptake (Varotto et al. 2002). Both transporters belong to the zinc-regulated transporter iron-regulated transporter like protein family (ZIP) that takes its name from the first transporter that was identified, the zinc regulated transporter ZRT. Indeed IRT1 and IRT2 are not highly specific for Fe and can mediate the import of a broad spectrum of metal species including zinc Zn^{2+}, manganese Mn^{2+} and cadmium Cd^{2+}. The *Arabidopsis thaliana* genome encodes for 16 ZIP proteins (Mäser et al. 2001) and they function in the uptake of different bivalent metal ions.

IRT1 is regulated at different levels. Its expression promptly responded after the plants were transferred to Fe deficiency (Connolly et al. 2002). In particular, the mRNA accumulated after 24 h and the protein level peaked after 72 h. After Fe resupply the IRT1 protein was already almost undetectable after 12 h, indicating that its expression is tightly regulated. An over-expressing line of *IRT1* showed accumulation of the protein only under Fe deficiency, indicating a fine regulation of Fe homeostasis at the uptake level. Indeed IRT1 protein is rapidly degraded in response to changing Fe conditions and this degradation is mediated by ubiquitination (Kerkeb et al. 2008; Barberon et al. 2011). Recently the specific E3 ubiquitin ligase IRT1 DEGRADATION FACTOR 1 (IDF1) was identified in a screen of insertional mutants (Shin et al. 2013).

The main feature of Strategy II is the excretion of chelating compounds such as mugineic acids (MAs) in the root rhizosphere that chelate Fe^{3+}, to enhance solubility and allow for mobilisation of Fe^{3+} (Fig. 5.1). The name mugineic acid is derived from the Japanese word komugi for wheat from which these compounds had first been isolated. The MAs biosynthetic pathway is conserved among the Poaceae family, which comprises many of the most important food plants: rice, wheat and maize, and starts from *S*-adenosyl-L-methionine (SAM). Three molecules of SAM are converted into nicotianamine (NA) in one reaction by nicotianamine synthase (NAS). NA is a precursor for MAs in strategy II plants but additionally functions as Fe chelator in different plant organs, and seems to be essential not only for the uptake of Fe from the soil, but also for the cell-to-cell and long distance transport within the plant (Schuler et al. 2012). Studies on NA started with the analysis of the tomato mutant *chloronerva*. This mutant is NA-free and shows retarded growth and intercostal chlorosis of young leaves. Map-based cloning revealed that chloronerva is a single copy gene in tomato and encodes for NAS (Ling et al. 1999). The NAS genes have been cloned from other plant species such as barley, rice and *Arabidopsis*, showing that NA carries out functions in strategy I and II species (reviewed by Hell and Stephan 2003; Klatte et al. 2009). The

expression of most NAS genes is strongly induced upon Fe deficiency. NA is then further processed by NA aminotransferase (NAAT) and deoxymugineic acid synthase (DMAS) to form 2′-deoxymugineic acid (DMA). DMA is the starting point for the synthesis of all the other chemical forms of MAs (Nakanishi et al. 2000). The secretion of MAs is diurnally regulated, with a peak in the morning (Cakmak et al. 1998). The transporter of mugineic acid family phytosiderophores 1 (TOM) has been identified in rice and barley as responsible for the secretion of MAs (Nozoye et al. 2011). After secretion, MAs can bind to Fe^{3+} and the MA-Fe^{3+} complexes are taken up by the root YELLOW STRIPE 1 (YS1) and YELLOW STRIPE 1-like transporters (YSL1) (Curie et al. 2009; Inoue et al. 2009). The study of these transporters started with the analysis of the maize mutant *yellow stripe 1*. This mutant shows leaf chlorosis and fails to take up phytosiderophores from the soil (Von Wiren et al. 1994). The *YELLOW STRIPE 1* gene was then mapped and cloned (Curie et al. 2001) and found to encode for a membrane transporter that mediates the uptake of phytosiderophores bound to Fe^{3+}. The biochemical function is directly shown by the ability of YS1 to restore the growth of a yeast strain deficient in Fe uptake, when phytosiderophores are present in the media. Its mRNA accumulates significantly under Fe deficiency in both root and shoot. The latter was surprising as phytosiderophores were not expected to be transported in green tissues as they are only present in the root (Curie et al. 2001). Indeed, although the release of phytosiderophores is a prerogative of graminaceous plants, YSL transporters are found also in non-graminaceous taxa (Chu et al. 2010, 2013). In particular, *Arabidopsis thaliana* has eight YSL genes (Mäser et al. 2001). In addition, YSL transporters have been found to transport NA-Fe^{3+} complexes (reviewed by Chu et al. 2013), adding to the fundamental role of NA as Fe chelator in both strategy I and strategy II plants.

Interestingly, rice additionally possesses an iron transporter, OsIRT1, an ortholog of AtIRT1. However, rice roots show a very low ferric-chelate reductase activity, suggesting a role of OsIRT1 in the direct uptake of Fe^{2+} in anaerobic growth conditions that is typical for this crop (Ishimaru et al. 2006).

Both graminaceous and non-graminaceous plants possess other divalent metal transporters, which can facilitate Fe assimilation. The NRAMP (natural resistance-associated macrophage protein) family transporters have been first found in mammals and have then been cloned from *Arabidopsis thaliana* (Curie et al. 2000) There are six genes encoding for NRAMP proteins in *Arabidopsis* (Mäser et al. 2001). They can mediate the uptake of several divalent metals, including Fe^{2+}, zinc Zn^{2+}, manganese Mn^{2+}, nickel Ni^{2+} and cadmium Cd^{2+}. The expression of three of these transporters, NRAMP1, NRAMP2 and NRAMP4, is induced by Fe deficiency in roots and leaves. In particular, NRAMP1 is thought to mediate the uptake of Fe and other essential nutrients, such as manganese from the soil (Curie et al. 2000; Cailliatte et al. 2010). The other two transporters NRAMP3 and NRAMP4 are involved in the Fe distribution to developing seeds in low Fe conditions (Lanquar et al. 2005).

Regulation of the Strategies

The regulation of Fe deficiency responses is very complex and requires the coordination of several regulatory elements. The presence of different pathway and feedback signals constitute an important aspect in the regulation.

Several transcription factors that are involved in the regulation of the Fe uptake machinery have been already identified in different Strategy I plants (Fig. 5.2a). The key element/regulator in this respect was first identified in the tomato *fer* mutant. Map-based cloning revealed that *FER* encodes for a basic helix-loop-helix (bHLH) transcription factor (Ling et al. 2002). *Arabidopsis* possesses an ortholog of *FER*, which has been named *FIT* (*FER-like iron deficiency-induced transcription factor*, also named before *FIT1/FRU/AtbHLH029*; Bauer et al. 2007). *FIT* expression is repressed upon full Fe supply, whereas the expression is highly induced in Fe deficiency. FIT positively regulates the expression of different Fe-responsive genes, including *IRT1* and *FRO2*. The *fit* mutant shows leaf chlorosis and decreased Fe content and fails to induce the typical Strategy I responses (Colangelo and Guerinot 2004). Moreover, the mutant dies at the seedling stage unless watered with additional Fe. *FRO2* mRNA level is severely downregulated in the mutant and the FRO activity cannot be detected. The transcript level of *IRT1* is decreased but still detectable in *fit* plants whereas the protein IRT1 is not present. These results suggest that FIT can control the Strategy I responses at different level, regulating the gene expression but also the turnover of IRT1 protein.

FIT can also interact directly with other bHLH factors, such as bHLH038 and bHLH039. This interaction is thought to serve in modulating the plant response to Fe deficiency (Yuan et al. 2008). These two factors belong together with bHLH100 and bHLH101 to a specific sub-group of bHLH and their expression is strongly induced upon Fe deficiency (Wang et al. 2007). They are also functioning independently from FIT to mediate Fe deficiency responses (Sivitz et al. 2012).

FIT can also directly interact with ETHYLENE INSENSITIVE 3 and ETHYLENE INSENSITIVE 3–LIKE1 (Lingam et al. 2011). This interaction provides the molecular link between ethylene and the responses to Fe starvation, which was elusive before. Ethylene is known to be a positive regulator of the induction of different Fe responsive genes. The ethylene downstream transcription factors EIN3 and EIL1 are required for FIT accumulation and therefore thought to inhibit its proteasomal degradation, thus enhancing the plant responses to Fe deficiency (Lingam et al. 2011).

Microarray analysis aimed at finding new regulatory candidates identified the bHLH transcription factor POPEYE (PYE) (Long et al. 2010). PYE is upregulated specifically in the cells of the root perycicle upon Fe deficiency. The mutant *pye* displays severely impaired growth under – Fe condition; therefore PYE seems to play a fundamental role in the roots of plants exposed to Fe deficiency. Moreover, PYE is proposed to negatively regulate a cluster of Fe-responsive genes, amongst these *NAS4* and *FRO3*. PYE can directly interact with PYE homologues, such as IAA-Leu Resistant3 (ILR3) and bHLH115. ILR3 in turn interacts with another
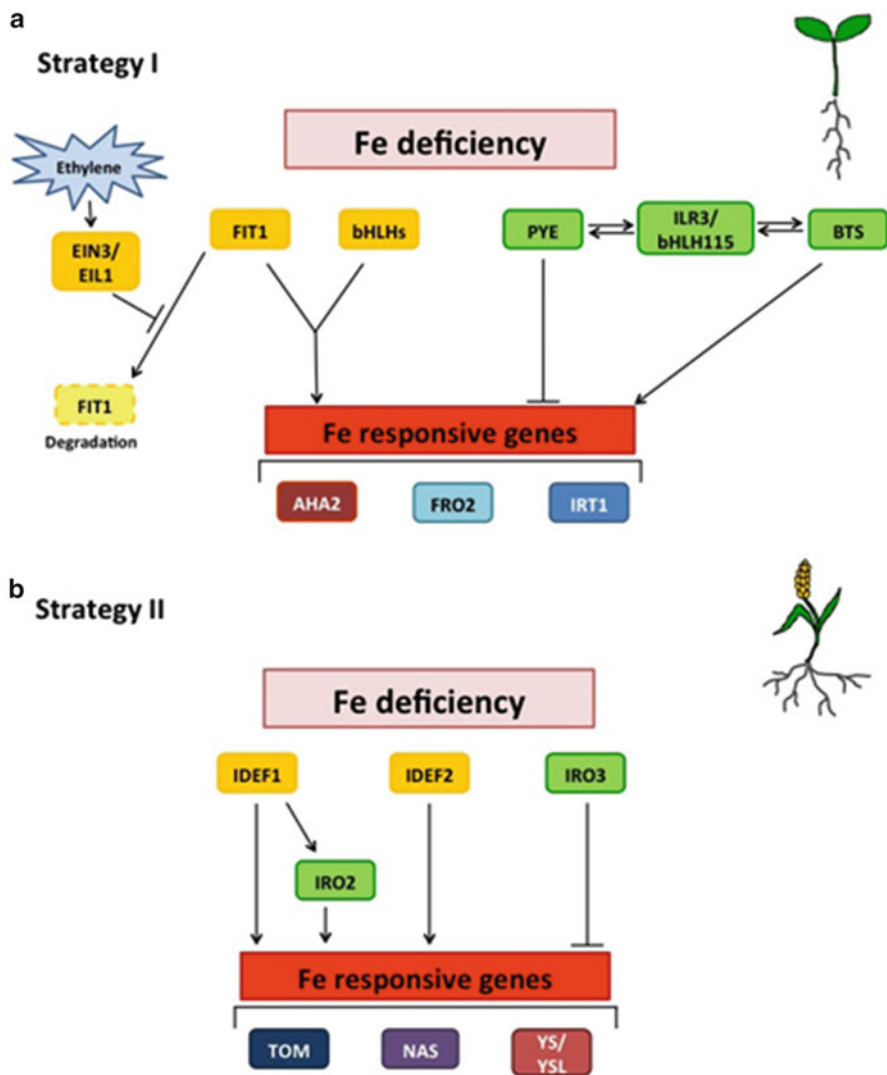
Fig. 5.2 Regulation of Fe deficiency responses in (**a**) Strategy I and (**b**) Strategy II plants. *Rectangles* indicate important regulatory transcription factors of the two Strategies and downstream Fe responsive genes. *Arrows* indicate positive or negative regulation. Abbreviations: *bHLH* basic helix-loop-helix, *FIT* FER-like iron deficiency induced, *EIN3/EIL1* ETHYLENE INSENSITIVE 3/ETHYLENE INSENSITIVE 3–LIKE, *PYE* POPEYE, ILR3, *BTS* BRUTUS, *IDEF* iron deficiency responsive element-binding factor, *IRO* iron-related transcription factor

regulatory protein named BRUTUS (BTS). BTS possesses three different domains, one with putative E3 ligase activity, one for transcriptional regulation and one for Fe binding. Unlike *pye*, *bts* mutants appear more resistant to Fe deprivation and show a better growth in – Fe conditions, with longer roots and greener shoots. A

direct interaction between PYE and BTS has not been reported, but interestingly BTS interacts with the PYE interactors ILR3 and bHLH115. It is therefore speculated that this interaction participates in the regulation of Fe deficiency responses in the root. The induction of PYE under Fe limiting conditions might serve to regulate Fe homeostasis in the plant. Additionally BTS, the antagonist of PYE, might help in this regulation controlling PYE activity (Long et al. 2010).

Other regulatory elements have been identified in Strategy II plants (Fig. 5.2b). The analysis was based on stepwise promoter analysis of the barley *IDS2* gene in tobacco and allowed the identification of two key regulators of Fe deficiency responses, the *cis*-acting iron deficiency responsive element 1 (IDE1) and IDE2 (Kobayashi et al. 2003). IDE1 and IDE2 were the first discovered *cis*-acting elements related to nutrient deficiency. From sequence alignment of the promoters of several Fe responsive genes it emerged that these *cis*-elements are quite conserved among different plant species. Indeed they have been found in several genes, *e.g.* *HvNAAT*, *HvNAS*, *OsNAS2*, *OsNAS3*, *OsIRT1*, *AtIRT1* and *AtFRO2*. IDE1 and IDE2 can interact with two rice transcription factors IDE-binding factor 1 (IDEF1) and IDEF2 (Kobayashi et al. 2007; Ogo et al. 2008). These two factors are members of the ABI3/VP1 (ABSCISIC ACID INSENSITIVE 3/VIVIPAROUS 1) family and NAC (NO APICAL MERISTEM, *Arabidopsis* transcription activation factor and CUP SHAPED COTYLEDON) family, respectively. IDEF1 and IDEF2 are constitutively expressed in vegetative tissues and can regulate two different sets of genes (Kobayashi et al. 2009). IDE1 regulates most of the Fe related genes in normal Fe conditions and during the early responses to Fe deficiency. Interestingly, IDEF1 can switch its target genes in the late stages of Fe deficiency. IDEF2 instead maintains the same target genes during the responses to Fe deficiency and it is known to positively regulate the expression of *OsYSL2* (Kobayashi et al. 2010). Therefore, IDEF2 is also involved in the correct partitioning of Fe between roots and shoot.

Many regulators from graminaceous plants have been identified by a microarray analysis approach. The most extensively studied candidate is *OsIRO2*, which encodes a bHLH transcription factor (Ogo et al. 2011). Its expression is positively regulated by IDEF1 in Fe deficiency. OsIRO2 can in turn positively regulate different Strategy II genes, such as *OsNAS1*, *OsNAS2*, *OsNAAT1*, *TOM1* and *OsYSL15*. Another bHLH transcription factor, *OsIRO3*, is present in rice and its expression is induced by Fe deficiency (Zheng et al. 2010). It seems to be a negative regulator of several genes related to Fe deficiency responses.

Intriguingly, sequence comparison with *Arabidopsis* transcription factors has shown that OsIRO2 is similar to AtbHLH038, 039, 100 and 101, whereas IRO3 is similar to PYE (Ogo et al. 2006). Thus far, no correspondent of FIT has been found in graminaceous plants and no orthologue of IDEF1 and IDEF2 has been found in non-graminaceous plants. Therefore, it seems that the regulatory mechanisms are only partially conserved between Strategy I and Strategy II plants.

Apart from the positive regulator ethylene, other signaling molecules and plant hormones participate in the regulation of Fe deficiency responses. Among these nitric oxide (NO), carbon dioxide and auxin also contribute to the induction of

several Fe-responsive genes. In contrast, cytokinin and jasmonic acid can negatively regulate the expression of different Fe genes such as *IRT1*, *FRO2* and *FIT* (reviewed by Kobayashi and Nishizawa 2012).

While the regulation of the Fe deficiency responses has been elucidated, the Fe sensing mechanism in the root remains unidentified. Recently the IDEF1 transcription factor has been found to bind directly to Fe and other divalent metals via its proline-rich domains and histidine-asparagine residues (Kobayashi et al. 2012). Thus, this transcription factor might be one factor for the sensing of the actual Fe situation in the cell and thus the Fe availability.

Fe Homeostasis in the Cell

After uptake in the root, further steps are required in order to allocate Fe in the rest of the plant. Fe must first be transported from the root epidermis through the root tissues to be loaded into the xylem. Due to its low solubility, a symplastic transport is assumed, but little is known about the mechanism and possible carrier (Morrissey and Guerinot 2009). Once it reaches shoot tissues, other mechanisms must be involved for the unloading and the transport into the different cellular compartments.

The solubility problem requires that Fe must always be in a chelated form during transport within the plant. Another important reason for the chelation is the potential toxicity of free ionic Fe that can catalyse the formation of reactive oxygen species (ROS) causing cell damage. Citrate, NA and MAs are the known predominant Fe chelators. In particular, citrate plays a fundamental role in chelating Fe in the xylem. Indeed, citrate-Fe(III) complexes have been found in the xylem sap of tomato plants (Rellan-Alvarez et al. 2010). FERRIC REDUCTASE DEFECTIVE 3 (FRD3) is an *Arabidopsis* multidrug and toxin efflux (MATE) transporter that plays a fundamental role in balancing Fe homeostasis. Its orthologue, OsFRDL1, has been found in rice. Both transporters mediate the efflux of citrate into the xylem. The *frd3* mutant displays chlorotic and dwarf phenotype, constitutive up-regulation of the Fe deficiency genes and of FRO activity. A high level of Fe is found in the roots of the mutant, due to inefficient Fe translocation to the shoot, emphasising the importance of citrate for Fe transport from root to shoot (Durrett et al. 2007; Rogers and Guerinot 2002). As FRD3 and FRDL1 efflux citrate in the Fe-free form, other transporters must be involved in the transport of Fe into the xylem. The *Arabidopsis* ferroportin 1/iron regulated 1 (AtFPN/AtREG1) could be involved in this process. Although direct evidence is still lacking, the localisation, the promoter activity and the mutant phenotype make this transporter a promising candidate (Morrissey et al. 2009).

Other transporters are involved in unloading the xylem into phloem. Members of the YSL family are widely expressed in different tissues also of non-graminaceous plants, suggesting a role in Fe translocation from xylem to phloem besides the uptake of MA-Fe complexes from the soil. Indeed YSL transporters have been

found to mediate the transport of NA-Fe complexes (Curie et al. 2009). The *Arabidopsis* YSL1 and YSL2 proteins were found to localise to the plasma membrane and to function in yeast complementation assay (Chu et al. 2010). They are active in leaves and in flowers and are therefore required for the fertility and the development of seeds and for the distribution of Fe to the seeds. The two transporters and *AtYSL3* are quite closely related but they have distinct functions in the plant, as neither *YSL1* nor *YSL2* under control of *YSL3* promoter could complement the double mutant *ysl1ysl3*. YSL4 and YSL6 were found to localise to the chloroplast (Divol et al. 2013), to the tonoplast and to internal membranes (Chu et al. 2013). They are thought to mediate the release of Fe from the chloroplast in case of Fe overload, thus controlling Fe homeostasis.

NA certainly represents the principal Fe chelator in the cell for different reasons (reviewed by Hell and Stephan 2003). It can form complexes with both Fe^{3+} and Fe^{2+} at neutral and basic pH, NA-Fe complexes are unlikely to react with oxygen in the Fenton reaction, NA is found in all plant tissues and also all plant species its concentration positively correlates with the root areas of Fe uptake. NA is also involved in loading the seeds with Fe (Klatte et al. 2009). It is, however, also able to bind and transport other transition metals such as zinc (Haydon et al. 2012).

Partitioning of Fe in the Organelles

The partitioning of Fe to the organelles must be tightly regulated, due to the high requirement for the biosynthesis of Fe-S clusters in both chloroplasts and mitochondria. In addition, synthesis of cytosolic Fe-S depends on provision of a precursor from the mitochondria (Balk and Pilon 2011). The import of Fe into the chloroplast also represents an important strategy to store Fe in a non-toxic and available form. Indeed the largest amount of Fe in plant cells is found in the chloroplast, where 80–90 % of Fe is accumulated (Marschner 1995). The members of the ferritin family (FER) play a fundamental role to prevent oxidative damage in case of Fe overload. Ferritins are spherical protein complexes formed by 24 subunits. They can internalise Fe atoms in their central cavity and can release them when needed (Briat et al. 2010). Animal ferritins are regulated mostly at the translational level, while phytoferritins are mainly subjected to transcriptional regulation.

In *Arabidopsis*, there are four ferritin isoforms (FER1, FER2, FER3 and FER4). A loss-of-function approach was used to investigate the role of this protein in different plant tissues (Ravet et al. 2009). The analysis showed that plants lacking ferritins were more sensitive to excess of Fe, with reduced growth and defects in flower development. Moreover, loss-of-function mutant plants presented differential regulation of genes related to Fe uptake and higher level of ROS and consequently higher activity of detoxifying enzymes. Electron microscopy has shown that plant ferritins localise to the plastids, mainly to non-photosynthetic ones such as proplastids, etioplasts and amyloplasts (Seckback 1982). The loss-of-function

approach provided more evidence that ferritins are not actually required for the proper formation of the photosynthetic chloroplast or for the functioning of the photosynthetic apparatus. Indeed, ferritins seem to play a fundamental role in the protection against oxidative stress (Ravet et al. 2009).

Other studies have attempted to further elucidate the localisation of ferritins in plant cells.

According to Zancani et al. (2004) ferritins can also localise to the mitochondria. Indeed, according to bioinformatics analyses, the *Arabidopsis* AtFER4 is the isoform most likely to be targeted to the mitochondria. A study was conducted based on the knock-out mutant *atfer4*. An antibody against FER was applied to protein fractions of isolated mitochondria and a ferritin signal was found in mitochondria isolated from wild type plants subjected to high Fe supply. The signal was not present in the fraction isolated from the mutant plants. This mitochondrial isoform seems to be of great importance for balancing Fe homeostasis in hetero-trophic tissues, as shown by work on suspension cell cultures (Tarantino et al. 2010). Petit et al. (2001) identified a *cis*-element in the region of maize ferritin gene *ZmFER1* and in its orthologue from *Arabidopsis AtFER1*. This regulator named iron-dependent regulatory sequence (IDRS) is able to repress the transcription of the gene under low Fe conditions. IDRS also has additional functions in *Arabidopsis*, where it triggers the expression of *AtFER1* under dark-induced senescence but not in age-dependent senescence and in seedlings (Tarantino et al. 2003). This suggests that more regulatory elements must be involved in the regulation of *AtFER1* expression under such conditions.

Time for coffee (TIC) has been found in a luciferase-based genetic screen of the *AtFER1* promoter (Duc et al. 2009). TIC has been previously described as a nuclear component of the circadian clock. Mutants of TIC are chlorotic unless supplied with exogenous iron and are hypersensitive to iron during the early stages of development. Thus TIC is a central regulator of *AtFER1* as it represses its expression in low Fe conditions, in a way that is independent from IDRS. The *tic* mutants also fail to repress other genes induced by Fe overload under low Fe, pointing out that TIC-dependent pathways are fundamental for the response to Fe overload.

Another Fe binding protein, which has been reported to be involved in the protection against photo-oxidative damage is frataxin. This protein has been hypothesised to participate in the mitochondrial biosynthesis of Fe-S cluster acting as Fe donor. Its importance for plant cell has been demonstrated by analysis of T-DNA insertion mutants in *Arabidopsis*. Indeed frataxin knock-out mutants are lethal, while the knockdown ones are viable but accumulate high levels of ROS and induce the expression of genes encoding for ROS scavenging proteins (Busi et al. 2006).

The import of Fe into the chloroplast is linked to ferritin function. The permease in chloroplast 1 (PIC1) was first identified as member of the chloroplast inner membrane translocon complex TIC (Teng et al. 2006). This transporter emerged as a possible candidate in a bioinformatic screening for the Fe importer in the chloroplast proteome, due to its biochemical characteristics such as hydrophobicity, basic isoelectric point and predicted transmembrane domains, and was therefore

renamed permease in chloroplast 1 PIC1 (Duy et al. 2007). The loss-of-function mutants of PIC1 display chlorotic phenotype and severely impaired growth. Moreover, the mutation causes severe problems in chloroplast development and leads to disturbed metal homeostasis in leaves.

FRO7, one of the members of the *Arabidopsis* FRO family, localises to the inner chloroplast membrane and it is thought to be essential for seedling development. The growth of the fro7 mutant is significantly impaired in alkaline conditions and in media lacking sugar. Moreover, chloroplasts isolated from the mutant exhibit significantly lower FRO activity and Fe content compared to the wild type (Jeong et al. 2008). These results suggested an important role of FRO7 as a chloroplast Fe transporter during photosynthesis and development.

In addition, the mitochondria contribute to cellular Fe homeostasis. Indeed a mutation in the STARIK/ATM3 gene that encodes for a mitochondria ABC transporter leads to chlorosis and reduced growth. The mitochondria of the *starik* mutant accumulate more non-heme and non-protein bound Fe, as the biosynthesis of Fe-S clusters in the mitochondria is linked to the intracellular Fe by this transporter (Kushnir et al. 2001; Bernard et al. 2009). A mitochondria iron transporter (MIT) has been identified in a screening of T-DNA (transfer DNA) of rice in Fe deficiency conditions (Bashir et al. 2011). The homozygous knock-out mutant *mit* is lethal, highlighting the importance of this transporter for plant growth. In contrast, the heterozygous mutant is viable but severely impaired in growth, exhibiting an accumulation of Fe in the shoot with less Fe in the mitochondria.

The vacuole also functions to store Fe and avoid toxicity. The vacuolar iron transporter (VIT) mediates the transport of Fe from the cytosol into the vacuole and plays a fundamental role in seed and seedling development, as shown by the analysis of the *vit1-1* mutant (Kim et al. 2006). In contrast, NRAMP3 and NRAMP4 are influx transporters that function to export Fe from the vacuole into the cytosol during seed germination (Lanquar et al. 2005). Their contribution is essential to provide Fe during development until the seedlings can start to take up Fe from the environment.

Interaction of Fe and S Metabolism (Uptake, Fe-S Clusters)

Uptake and homeostasis of Fe is known to be linked to other metals such as zinc (Lin et al. 2009; Deinlein et al. 2012). More recently the connection between Fe and sulfur (S) metabolism is being recognised, mainly because Fe is required together with S for the biosynthesis of the Fe-S clusters.

The thylakoids in chloroplasts harbour ferredoxin, photosystem I (PSI) and cytochrome b_6f complex, which belong to the photosynthetic electron transport chain. In the stroma, other Fe-S proteins are found; among these there are nitrite reductase and two key enzymes for sulfur metabolism, sulfite reductase and APR. In mitochondria, major Fe-S proteins are Complex I, II and III of the respiratory

chain and aconitase. Other Fe-S proteins are found in the nucleus and function in DNA replication (Balk and Pilon 2011) and damage repair (Liu et al. 2003).

The coordination of the plant's demand for Fe-S clusters supports the hypothesis of a co-evolution between the Fe and the S metabolisms and the development of interaction traits between them.

Chelated Fe and reduced S in form of cysteine represent the substrates of the biosynthetic pathway. The Fe donor molecule is not known yet, whereas it is known that sulfur is mobilised from cysteine by a cysteine desulfurase. The protein frataxin has received attention for its putative role as Fe donor in the mitochondria assembly pathway (Busi et al. 2006).

As sulfur is present as acid-labile sulfide (S^{-2}) in the Fe-S cluster, two additional electrons are needed to reduce elemental sulfur S^0 to S^{-2} (Lill 2009). In the first step of the pathway, the Fe-S cluster is assembled on scaffold proteins. In the second step, the cluster is transferred to the specific apoprotein, which provides free amino acidic residues to bind it. Additional carrier proteins are involved in this step. The assembly machineries have been characterised in plants: chloroplasts contain the ISC (iron-sulfur cluster) biosynthetic pathway, whilst mitochondria contain the SUF-like (sulfur mobilization) pathway and in the cytoplasm the CIA (cytosolic iron-sulfur cluster assembly) pathway (reviewed by Balk and Pilon 2011; Couturier et al. 2013). The CIA is dependent on the mitochondria SUF assembly machinery, which provided the sulfide-containing compound that is used for the biosynthesis of the cluster. The mitochondrial ABC transporter, STARIK/ATM3, is thought to be involved in this process. Indeed the mutant *atm3* shows severely impaired activity of cytosolic aconitase while the mitochondria and plastidic isoforms are unaffected. Furthermore, *atm3* does not accumulate Fe in the mitochondria and the general Fe homeostasis is not affected (Bernard et al. 2009).

Upon nutrient deficiency, a complex reprogramming of cell metabolism occurs, in order to maintain viability. The strong requirement of Fe and S for the biosynthesis of Fe-S clusters in the organelles might constitute a feedback signal for the co-regulation of the assimilation pathways. Indeed the existence of such signals has been recently proposed for Fe and S metabolism (Vigani et al. 2013; Chan et al. 2013). The plant responses triggered by Fe or S deficiency have been well characterised. The consequences of the combined shortage of these nutrients however have only seldom been investigated, and might impact particularly on Fe-S cluster assembly. Significant interactions between Fe uptake mechanisms and external sulfate supply have been reported. It has been shown that S deficiency limits the capacity to cope with Fe shortage in tomato plants, preventing the expression of Fe chelate reductase *FRO1* and reducing the activity of Fe^{2+} transporter (Zuchi et al. 2009). Other studies using barley plants reported a positive correlation between S supply in the growth media and the plant capability of coping with Fe deficiency. Indeed phytosiderophores represent another important junction between Fe and S metabolism, as they are derived from the S-containing amino acid methionine. The release rate of phytosiderophores was diminished in barley plants upon sulfate deficiency, due to a decrease of the methionine level. After sulfate resupply, plants increased the release of phytosiderophores when exposed to Fe

shortage (Astolfi et al. 2010, 2012). Recently the effect of Fe deficiency on sulfur metabolism has been analyzed in durum wheat (Ciaffi et al. 2013). Wheat plants grown under sufficient S supply showed an up-regulation of certain S deficiency responses when exposed to Fe deficiency. The expression of the high affinity sulfate transporters was increased in the root, as well as of several genes of the S metabolic pathway.

Recently we found that also in *Arabidopsis thaliana* the expression of the two key genes for the uptake of Fe (*IRT1*) and of S (*SULTR1;1*) correlates with the supply of both Fe and S in the growth media. The expression is differentially regulated in case of double nutrient shortage (Forieri et al. 2013). We suggested that Fe-S cluster availability might function in sensing and signalling of combined Fe and S deficiencies. Altogether, these analyses strongly support the existence of a co-regulation between the metabolic pathways, as the limitation of one nutrient influences the uptake of the other one. Such a co-regulation is very likely to be the outcome of a complex remodelling of the whole plant metabolism upon nutrient limitation as known for the prolonged deficiency of the single nutrients (Schuler et al. 2011; Nikiforova et al. 2003). Hence, we propose different signals that might contribute to this co-regulation, such as the sensing of the Fe and S concentrations in the root rhizosphere or within root cells, ROS, metabolism intermediates, Fe-S cluster assembly machineries or Fe-S proteins.

Conclusions

Fe is one of the most fascinating elements for life functions due to its redox properties and is of great importance for human nutrition. Crop plants are the direct or indirect source of Fe in our food, and research on the dicot model plant *Arabidopsis thaliana* and also on rice has provided tremendous advances in our understanding of plant Fe homeostasis in the past years. In particular, the primary uptake processes into the root are now based on molecular evidence for the genes involved in Strategy I (reduction based) and Strategy II (chelation based). The allocation of Fe from the rhizodermis to xylem and phloem for supply of young and growing tissues and recirculation to roots is, however, much less well understood. Finally, transport processes inside cells are beginning to be unraveled, explaining how iron homeostasis is mediated between cytosol, plastids, mitochondria and the vacuole. The key genes involved in these processes also represent possible candidates in the search for Fe use efficient plants.

A crucial process in homeostatic control is the chelation of the almost insoluble and redox active free Fe ions. Fe chelated by nicotianamine is carried across plasmalemma and endomembranes and also for long distance transport. Future work needs to address the mechanisms of donation of Fe from chelators to acceptor molecules such as heme, Fe-S clusters and proteins. In addition, the regulatory networks of transcription factors that

function in the sensing of Fe deficiency, adaptation of root morphology and coordination with uptake of other nutrients are only beginning to be discovered. Recent observations indicate a co-regulation of Fe homeostasis with the uptake and metabolism of S, most likely triggered by the demand for Fe-S clusters in the electron transport chains of plastids and mitochondria. Detailed understanding of Fe homeostasis is prerequisite for the generation of Fe efficient plants and enhanced Fe contents in food.

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