Essential and Detrimental - An Update on Intracellular Iron Trafficking and Homeostasis

Abstract
Chloroplasts, mitochondria and vacuoles represent characteristic organelles of the plant cell, with a predominant function in cellular metabolism. Chloroplasts are the site of photosynthesis and therefore basic and essential for photoautotrophic growth of plants. Mitochondria produce energy during respiration and vacuoles act as internal waste and storage compartments. Moreover, chloroplasts and mitochondria are sites for the biosynthesis of various compounds of primary and secondary metabolism. For photosynthesis and energy generation, the internal membranes of chloroplasts and mitochondria are equipped with electron transport chains. To perform proper electron transfer and several biosynthetic functions, both organelles contain transition metals and here iron is by far the most abundant. Although iron is thus essential for plant growth and development, it becomes toxic when present in excess and/or in its free, ionic form. The harmful effect of the latter is caused by the generation of oxidative stress. As a consequence, iron transport and homeostasis have to be tightly controlled during plant growth and development. In addition to the corresponding transport and homeostasis proteins, the vacuole plays an important role as an intracellular iron storage and release compartment at certain developmental stages. In this review, we will summarize current knowledge on iron transport and homeostasis in chloroplasts, mitochondria and vacuoles. In addition, we aim to integrate the physiological impact of intracellular iron homeostasis on cellular and developmental processes.
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Introduction

In the plant cell, chloroplasts and mitochondria perform photosynthetic and respiratory electron transport across their thylakoid and inner membrane systems, respectively. To fulfill these tasks, both organelles contain the transition metals iron (Fe), copper (Cu) and manganese (Mn), which due to their potential for valency changes are essential for functional electron transfer chains (Raven et al. 1999). However, performance of oxygenic photosynthesis and respiration in the plant cell require strict control of transition metal transport and homeostasis since metal-catalyzed generation of reactive oxygen species (ROS) causes oxidative damage. This is most acute in chloroplasts and mitochondria, where radicals and transition metals are located in close proximity and ROS production is a usual feature of electron transport and the water-splitting apparatus. Thus, on the one hand metals are a prerequisite for photoautotrophic life when bound by proteins or part of a chelate complex, but on the other hand they become toxic when present in their highly reactive, radical generating, free ionic forms. As a consequence, transport, storage and cofactor-assembly of metal ions have to be tightly controlled and are crucial during plant growth and development (Thomine and Vert 2013). An internal reservoir and storage compartment for transition metals in plant cells, however, is provided by the vacuole. Here, reactive metal ions can easily be stored when toxic and delivered for physiologically important processes by controlled loading and release via specialized transport proteins (Peng and Gong 2014).

In recent years, several membrane proteins for the transport and storage of transition metals have been discovered in chloroplasts, mitochondria and vacuoles. In parallel, physiological measurements for metal transport in intact, isolated organelles have been developed. In this review, we will thus focus on the description of Fe transport and storage in these three organelles as well as their implications and integration into cellular physiology and metal homeostasis mainly in leaf (chloroplast, mitochondrion) and seed (vacuole) tissues. For Fe transport across other membranes as well as for processes in roots and symbiosomes, we suggest readers to refer to recent

reviews (Brear et al. 2013, Clarke et al. 2014, Connorton et al. 2017, Curie and Mari 2017, Jeong et al. 2017, Kobayashi et al. 2019). For a general and tissue-specific subcellular distribution of transition metals, see Kim et al. (2006), Mary et al. (2015), Roschzttardtz et al. (2013), and Yruela (2013).

Chloroplasts and Iron

Chloroplasts originated about 3 billion years ago by the endosymbiosis of an ancestor of today's cyanobacteria with a mitochondria-containing host cell (Gould et al. 2008, Zimorski et al. 2014). In present-day land plants, organelles of the plastid family perform essential functions of primary and secondary metabolism that require an extensive exchange and transport of metabolites and ions with the surrounding cellular environment. Above all, however, chloroplasts are the site of photosynthesis and thus represent the basis for all life dependent on atmospheric oxygen and carbohydrate supply. In total, the photosynthetic electron transport chain requires a ratio of 22 Fe, 4 Mn and 1 Cu ions for proper function (Yruela 2013). Thus, iron is by far the most abundant transition metal of the photosynthetic apparatus and is present in all electron transfer complexes [photosystem (PS) II and I, cytochrome b6f complex, and ferredoxin]. In addition, in chloroplasts, Fe is required for the biogenesis of cofactors such as heme and Fe–S clusters, and Fe-cofactor containing proteins are involved in protein import and manifold physiological processes as specified below (Balk and Schaedler 2014, Briat et al. 2015). Taking into consideration the classification of Fe-requiring enzymes (FeRE) (Vigani and Murgia 2018), several proteins belonging to almost all FeRE categories are localized in chloroplasts (Fig. 1). Indeed, chloroplast FeRE are involved in (i) O2-dependent electron transport chain (photosynthesis-related pathways); (ii) ROS scavenging (Fe-superoxide dismutase, peroxidase); (iii) chloroplast DNA metabolism; (iv) dioxygenases (e.g. enzymes for carotenoid biosynthetic pathways and fatty acid metabolism); (v) monooxygenases (carotenoid and tetrapyrrole biosynthetic pathways) and other Fe-dependent reductase enzymes for nitrogen and sulfate assimilation processes (Blaby-Haas and Merchant 2013, Vigani and Murgia 2018). As a consequence, chloroplasts represent the Fe-richest cellular compartment in plant cells containing 80–90% of the Fe found in leaf cells (Terry and Low 1982). In addition, the Fe content of plastids has an important regulatory role. Mature chloroplasts for example seem to be central hubs for circadian Fe sensing, since alongside light regulation by phytochrome photoreceptors, the circadian clock adjustment also depends on the Fe status of chloroplasts (Salome et al. 2013). Excess Fe, however, is detrimental due to the generation of ROS, which cause oxidative damage (Briat et al. 2010b). Free Fe ions for example lead to the formation of hydroxyl radicals via the Fenton reaction (Halliwell and Gutteridge 1992). In chloroplasts, this situation is most prominent since Fe and ROS, like hydrogen peroxide (H2O2), are produced by photosynthetic electron transport in close proximity (Asada 1999, Mubarakshina et al. 2010). In contrast, chloroplast Fe shortage impairs chlorophyll biosynthesis, leads to leaf chlorosis and requires remodeling of the photosynthetic apparatus (Spiller and Terry 1980, Moseley et al. 2002). Plastids suffering from Fe starvation are specifically impaired in proper function of PSI, which contains 12 Fe atoms per monomer. Thus, to maintain a balanced plastid Fe homeostasis and guarantee proper plant development and growth (Abadia et al. 2011, Briat et al. 2015), Fe uptake and export as well as storage and buffering of free Fe within this organelle need to be tightly controlled.

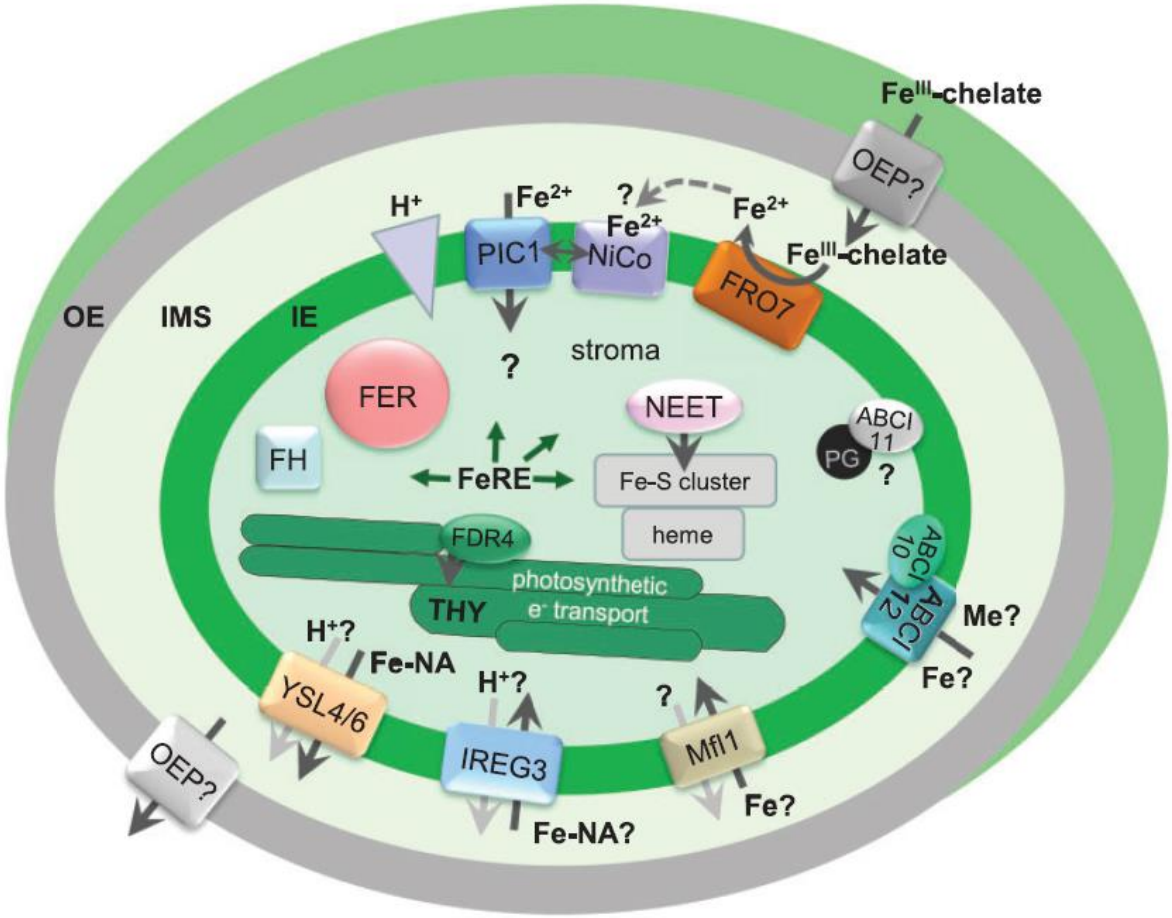

FIGURE 1 Iron transport and homeostasis in chloroplasts. Fe uptake across the chloroplast outer envelope membrane (OE) is proposed to occur in the form of FeIII-chelates. Most likely FeIII-citrate travels via a yet unknown ß-barrel OEP protein. At the inner envelope membrane (IE), the FCR FRO7 reduces the FeIII-chelate in the intermembrane space (IMS). Subsequently, Fe2+ might be bound by the membrane protein NiCo, which would initiate uptake by the permease PIC1. This transport at least in part could be dependent on the proton gradient across the IE (grey triangle). In the chloroplast stroma, iron is provided for Fe–S cluster and heme biogenesis as well as a cofactor for Fe-requiring enzymes (FeRE), which are involved in manifold chloroplast-intrinsic metabolic pathways. Whereas the protein NEET and the monocot specific FDR4 have been described to be involved in Fe transfer to the Fe–S cluster machinery and across thylakoid (THY) membranes, respectively, nothing is known about handover of iron after uptake. Also stroma-localized interaction partners for Fe transporters have not been described so far. Under Fe overload in mature chloroplasts and for storage in seed plastids, the ferritin (FER) protein shell acts as a buffer to prevent oxidative stress. Furthermore, the Fe-binding protein frataxin (FH), mainly found in the matrix of mitochondria (compare Fig. 2), might also contribute to plastid Fe storage. For Fe uptake, roles for IREG3/MAR1 as well as for the mitochondrial carrier family (MCF) protein Mfl1 have been described, which could provide alternative pathways to PIC1. However, since the generalized transport reaction for carriers of the MC family is an antiport mechanism (compare transporter classification database TCDB at http://www.tcdb.org), the counterion for Fe2+ transport by Mfl1 is still unknown. Iron for transport via IREG3/MAR1 might be chelated by nicotianamine (NA), the necessary H+-antiport mechanism of the ferroportin family is unclear. Fe-NA export most likely

occurs via the oligopeptide family transporters YSL4 and YSL6, however, this would probably require H+ symport as described for the oligopeptide transporter family. The Fe transport mediated by the potential PIC1/NiCo complex might be the main pathway in mature, photosynthetically active chloroplasts. Fe-NA traffic via IREG3/MAR1 and YSL4, YSL6 seems to be most important during seed development and leaf senescence or for Fe export (YSL6) at Fe excess. The gene ABCI11/NAP14, encoding a NBD-subunit of a prokaryotic-type, multisubunit ABC transporter complex is implicated in chloroplast Fe homeostasis. So far, protein interaction partners are unknown and it is unclear whether ABCI11/NAP14 associates with the IE or plastoglobuli (PG) or maybe both. Only very recently, the IE-intrinsic ABCI12 and the soluble NBD-subunit ABCI10/NAP13 have been described to be part of a novel, prokaryotic-type ECF ABC-transporter complex for metal transport, most likely uptake, in chloroplasts. Here ABCI10 would represent the ATP-binding subunit CbiO/EcfA, whereas ABCI12 resembles the membrane-intrinsic, energy-coupling subunit CbiQ/EcfT. Please note that except for PIC1, integration into the IE for all other potential Fe-transport proteins has not been unequivocally shown. For all membrane proteins the predicted number of α-helical transmembrane domains (α-TMD, Aramemnon database) (Schwacke et al. 2003), as well as the designated families according to TCBD (Saier et al. 2006) are as follows: ABCI12 (5 α-TMD, ABCI-subfamily ABC transporter, TC 3.A.1); FDR4 (2–4 α-TMD, specific for monocots); FRO7 (11 α-TMD); IREG3/MAR1 (11 α-TMD, ferroportin family, TC 2.A.100); Mfl1 (6 α-TMD, mitochondrial carrier family, TC 2.A.29, MFL-type); NiCo (6 α-TMD, Ni2+/Co2+ transporter family, TC 2.A.52); PIC1 (4 α-TMD, TC 2.A._PIC-type carrier); YSL4, YSL6 (14 α-TMD, oligopeptide transporter family, TC 2.A.67_YSL-type).

Fe homeostasis in chloroplasts

Ferritins (FER) are conserved eukaryotic proteins and the homo-oligomeric assembly of 24 subunits forms a hollow core that can store up to 4,000 Fe atoms, in chloroplasts most likely in the form of ferrihydrite (Fe3+)2O3·0.5H2O (for overview see Briat et al. 2010a, Briat et al. 2010b). The Arabidopsis nuclear genome contains four FER genes, which encode three proteins targeted to the stroma of leaf chloroplasts (Fig. 1), and one to seed plastids, respectively (Petit et al. 2001). Furthermore, plant FER have also been described in mitochondria (see below). In addition to their role as Fe-storage proteins during seed germination, chloroplast-intrinsic FER function in Fe sequestration under Fe overload. In contrast to seed tissue, FER proteins in mature leaf chloroplasts are of very low abundance but accumulate under Fe-excess conditions (Briat et al. 2010b) and thereby play an important role in buffering free Fe and preventing oxidative stress (Ravet et al. 2009a). Whereas in humans, the chaperone involved in Fe delivery to ferritin has been described (Shi et al. 2008), this machinery in plants is unknown (Briat et al. 2010a, Briat et al. 2010b). Due to the active redox nature of Fe, the involvement of metallo-chaperones or chelating molecules similar to the CCS chaperone, which delivers Cu to the Cu/Zn-superoxide dismutase in plastids is likely (Cohu et al. 2009). The mechanism underlying the release of Fe from ferritin is even more enigmatic. On the one hand, in vitro studies with animal FER suggest that this release requires Fe chelators or reducing agents. On the other hand, in vivo studies have demonstrated Fe release by proteolytic degradation of the protein (Briat et al. 2010a, Briat et al. 2010b). Given the observed correlations of PIC1 and YSL6 with ferritin function in leaf chloroplasts and seed plastids, respectively (see below), a role for transporters in the delivery or release of iron to or from the ferritin protein shell is likely.

Chloroplasts harbor an independent mechanism for the assembly of Fe–S clusters—the SUF (sulfur mobilization) pathway, which originated from their cyanobacterial ancestor (Balk and Pilon 2011).

While the identities of the desulfurase, several scaffold proteins, and cluster transfer and insertion proteins are known (Couturier et al. 2013, Balk and Schaedler 2014), it is still unclear which proteins deliver the Fe for Fe–S cluster formation. Here again, Fe-uptake transporters, which import the Fe to the stroma are likely to be important. A protein, which might play a role in Fe transfer inside chloroplasts, belongs to the NEET family (Fig. 1). NEET proteins contain a labile Fe2–S2 cluster, which allows a role in intracellular Fe–S/Fe transfer (Zuris et al. 2011). The Arabidopsis protein, At-NEET, is involved in Fe–S/Fe cluster transfer to an acceptor protein and initially was dually localized to the chloroplast and mitochondria (Nechushtai et al. 2012) but was described to be in the chloroplast stroma only later on (Su et al. 2013). At-NEET knockdown plants accumulate more Fe and have higher ROS levels than wild type (Nechushtai et al. 2012). The growth of At-NEET knockdown seedlings is insensitive to high, but sensitive to low Fe levels, strongly suggesting that this protein is involved in Fe transfer, distribution and/or management. Furthermore, At-NEET knockdown mutants present a dramatic decrease in catalase abundance, a heme enzyme that is localized in peroxisomes and detoxifies ROS, suggesting also a role of NEET in the supply of chloroplast heme Fe (Nechushtai et al. 2012).

Last but not least, the polyamine nicotianamine (NA) is a candidate to maintain Fe homeostasis in the chloroplast. NA has a role in metal chelating in phloem sap, vacuoles and cytoplasm (Pich et al. 1997, Haydon et al. 2012). Although the presence of NA in chloroplasts has not been directly demonstrated so far (Solti et al. 2012, Müller et al. 2019), in leaves from the tomato mutant chloronerva, which does not contain NA, electron dense inclusions of Fe and phosphor were observed in chloroplasts (Becker et al. 1995). Since these inclusions do not correspond to ferritin, these Fe precipitates might indicate a role of NA in maintaining iron in a soluble form in the chloroplast stroma. Further NA seems to play a role in chelate-based transport of Fe across the plastid envelope (see below and Conte and Lloyd 2010, Divol et al. 2013). Therefore, we postulate that some amounts of NA can be present in plastids, most likely under certain stress conditions and/or at distinct developmental stages.

Iron Transport in Chloroplasts

The Fe-uptake mechanisms of chloroplasts (Nouet et al. 2011, Lopez-Millan et al. 2016) are not as well-known as the two Fe-acquisition strategies occurring in roots, i.e. reduction-based Fe2+-transport (strategy I) and transport of chelated FeIII (strategy II) (see Morrissey and Guerinot 2009, Kobayashi and Nishizawa 2012, Brumbarova et al. 2015). In the cytoplasm of angiosperm cells, Fe is thought to form complexes to organic acids and chelators such as FeIII-citrate, FeIII-NA and FeII-NA (Weber et al. 2008, Bashir et al. 2016, Flis et al. 2016) that would be available for uptake by chloroplasts. Since various Fe complexes and chelates seem to be able to cross the chloroplast outer envelope (OE) membrane (Bughio et al. 1997, Solti et al. 2012, Müller et al. 2019), these compounds may also be present in the intermembrane space between the chloroplast OE and inner envelope (IE) membranes (Fig. 1).

Experiments with isolated, intact chloroplasts and purified IE membrane vesicles from barley (Hordeum vulgare) described the existence of a light-dependent uptake of 59FeIII, chelated by epihydro mugineic acid that was suggested to depend upon photosynthetic electron transport or ATP generated in thylakoids (Bughio et al. 1997). Chloroplasts of oilseed rape (Brassica napus) were shown to utilize stoichiometric FeIII–citrate, but not FeII–NA and FeIII–NA complexes. Additionally, these chloroplasts were unable to utilize iron of artificial FeIII-chelates for uptake (Müller et al. 2019). According to Mössbauer spectroscopy measurements, Fe uptake from an 57FeIII-citrate pool resulted in an accumulation of non-washable 57FeIII-citrate in sugar beet (Beta vulgaris)

chloroplasts that was thought to be the Fe pool accumulating between the two envelope membranes (Solti et al. 2012). Further, in sugar beet chloroplasts, the presence of free complexing ligands for ferrous iron (Fe2+) seemed to inhibit Fe uptake across the IE membrane (Solti et al. 2012). In chloroplasts from pea (Pisum sativum), however, an inward, strategy I-like Fe2+-transport across the IE was demonstrated (Shingles et al. 2001, Shingles et al. 2002). This Fe2+ uptake was inhibited by Zn2+, Cu2+ and Mn2+ in a competitive manner, and was activated by protons, similar to the reduction-based Fe-acquisition mechanism in roots.

Chloroplasts inherited a series of solute transporters from their prokaryotic ancestors (Tyra et al. 2007) including components of metal transport systems (Duy et al. 2007a, Shimoni-Shor et al. 2010, L. Voith von Voithenberg, J. Park, R. Stübe, Y. Lee and K. Philippar, unpublished work). Therefore, Fe-uptake mechanisms of prokaryotes, in particular that of Gram-negative bacteria (Braun and Hantke 2011, Lau et al. 2016), could serve as a blueprint for chloroplasts of land plants. Free living and photosynthesizing cyanobacteria are able to utilize either or both FeIII-siderophore complexes ("strategy II") and Fe2+ ("strategy I"). Across the outer membrane (OM), these cyanobacteria import FeIII-siderophore chelates or FeIII-oxides. At their plasma membrane, they either use a reduction-based uptake of Fe2+ by the FeoB transporter or directly transport oxidized Fe3+ bound to a periplasmic binding protein (FutA) via the Fut ABC (ATP-binding cassette) transporter complex (Braun and Hantke 2011, Kranzler et al. 2013, Kranzler et al. 2014, Rudolf et al. 2015). In the FutABC complex, FutC is a membrane-associated ATPase (NBD-subunit for nucleotide-binding-domain of ABC transporters) that energizes the FutB permease. In the freshwater cyanobacterium Synechocystis sp. PCC 6803, the uptake of free Fe2+ requires the presence of alternate respiratory terminal oxidase (ARTO). ARTO is a plasma membrane localized protein similar to cytochrome c oxidase that couples the respiratory electron transport chain to the reduction of Fe in the periplasmic space and thus can mediate the reduction-based Fe uptake of cyanobacterial cells (Kranzler et al. 2014). In Gram-negative Escherichia coli and also in cyanobacteria, Fe uptake across the OM occurs via receptor-gated ß-barrel channels also called TonB-dependent transporters (TBDTs), which transport FeIII-chelates and are energized by the TonB system at the plasma membrane (Kranzler et al. 2013, Braun 2014, Rudolf et al. 2015). One of the most prominent TBDT is the TonB-dependent FeIII-citrate receptor-gated channel FecA from E. coli that is energized by a proton gradient (Braun and Herrmann 2007, Marshall et al. 2009). Here the TonB system transfers the energy, originating from the polarization of the plasma membrane, to the FecA system (Braun 2014).

During photosynthesis, light-driven pH gradients are generated between the cytosolic site of chloroplasts (pH 7), the stroma (pH 8) and the thylakoid lumen (pH 6). Mostly ion channels and transporters in the IE and thylakoid membranes are involved in establishment of these pH gradients (Finazzi et al. 2015, Xu et al. 2015, Carraretto et al. 2016, Szabo and Spetea 2017). The trans-envelope voltage difference in chloroplasts in consequence is negative at the stromal site and most likely almost exclusively exists across the IE membrane. Here the steady-state membrane potential (positive at the face of the intermembrane space) is about −100 mV (Neuhaus and Wagner 2000). Across the OE it is proposed that no potential drop exists, except for a supposedly small Donnan potential difference due to accumulation of large weakly permeable anionic species in the intermembrane space (Pottosin and Dobrovinskaya 2015, Pottosin and Shabala 2016). In addition, the presence and transport of transition metal cations and oxoanions may change the polarization of the OE membrane due to their lower permeability compared to K+ and Cl-, which have relatively large ionic radius and thus a lower surface charge. This relatively low surface charge results in a smaller hydrate coat that may contribute to the higher mobility of K+ and Cl- across the OE. A similar

effect is explained for E. coli OMs by the action of the outer membrane matrix porin F (OmpF), a β-barrel subunit trimer, which forms wide aqueous multi-ion pores (Im and Roux 2002, Roux et al. 2004). Since this OE polarization was observed to alter the Fe uptake of chloroplasts, participation of a voltage-sensitive transport process was proposed for FeIII-complexes across the chloroplast OE (Solti et al. 2016). In addition, the Fe2+ movement via the chloroplast IE was also shown to be facilitated by a transmembrane electrochemical and proton gradient (Shingles et al. 2002).
In summary, physiological experiments indicate that chloroplast Fe transport across the OE membrane occurs most likely via FeIII-chelates, preferably FeIII-citrate. The main uptake pathway across the IE, however, seems to be in the form of non-chelated Fe2+ by a reduction-based and proton-driven mechanism similar to strategy I Fe uptake in roots (Fig. 1). Interestingly, a proposed plastid Fe export across the IE might take place via chelated FeII, mediated by YSL proteins (see below). These two different strategies for Fe uptake, diverging at the chloroplast OE and IE membranes are well in line with the situation in Gram-negative prokaryotes, however, other chelates and transport mechanisms are possible as well.

Chloroplast Fe-transport proteins

Due to their endosymbiotic origin, chloroplasts and mitochondria are unique as two membranes surround both organelles, similar to their Gram-negative prokaryotic ancestors. During evolution, the IE membrane of chloroplasts mainly derived from the bacterial plasma membrane. The chloroplast OE, however, largely originated from the OM of the Gram-negative cyanobacterial-like endosymbiont (Block et al. 2007). In the IE, numerous metabolite and ion transporter proteins have been characterized with respect to their physiological roles and molecular mechanisms (Weber and Linka 2011, Finazzi et al. 2015, Marchand et al. 2018). These channels and transporters are hydrophobic, mainly α-helical membrane proteins facilitating the exchange of ions, and metabolic products between plastids and the cytoplasm. In contrast, the characteristic channels of the OM in Gram-negative bacteria and chloroplast OE span the membrane in the form of β-strands that are organized to form a barrel-like pore structure (Duy et al. 2007a, Zeth and Thein 2010). In chloroplasts, these solute pores like the outer envelope proteins OEP21, OEP24, OEP37 and OEP40 (Pohlmeyer et al. 1998, Bölter et al. 1999, Goetze et al. 2006, Harsman et al. 2016) are essential parts of the OM permeom of these organelles (Breuers et al. 2011).
In the last decade, research on the molecular identity of chloroplast Fe-transporters suggests that several families of proteins may play a role in Fe uptake and export (for overview see Nouet et al. 2011, Finazzi et al. 2015, Lopez-Millan et al. 2016). Proteins that shuttle Fe across the OE have not been identified, nevertheless, they might be represented by ß-barrel OEPs (Fig. 1) similar to the ligand gated, TonB-dependent, ß-barrel receptor channels in the OM of Gram-negative bacteria, i.e. FecA for FeIII-citrate in E. coli or TBDTs in cyanobacteria (Chakraborty et al. 2007, Duy et al. 2007a, Kranzler et al. 2013). Unique experimental evidence for integration into the chloroplast IE membrane is only provided for the permease PIC1 (see below). However, several other potential Fe transporters, which have been localized to the chloroplast envelope, most likely are targeted to the IE membrane as well. How and if free Fe ions are transferred across the thylakoid membrane so far is unknown. Most likely is a previous assembly with proteins and cofactors of the photosynthetic electron transport chain in the stroma, followed by transfer of the holoprotein complex across the thylakoid membrane. An example is the assembled Rieske Fe/S-protein of the cytochrome b6/f complex that utilizes the TAT protein import pathway for its translocation into the thylakoid membrane (Molik et al. 2001). A possible, but still not experimentally documented function in the translocation of Fe-containing proteins across thylakoids has been assigned to the monocot specific

FDR3 and FDR4 from maize (Fig. 1) (Han et al. 2009, Zhang et al. 2017). In the following, we will focus on the latest results described for plastid Fe-transport and homeostasis proteins and their integration into cellular metal homeostasis. For a comprehensive overview of additional transporters, we refer to Lopez-Millan et al. (2016).

The Fe-uptake permease PIC1. Permease in chloroplasts 1 (PIC1) in Arabidopsis was the first protein, described to be involved in chloroplast Fe uptake (Fig. 1). For this integral membrane protein, four membrane-spanning α-helices were predicted. Insertion into the IE membrane of chloroplasts was clearly shown by in vivo green fluorescent protein (GFP) targeting and immunoblot analysis (Duy et al. 2007b). The function of PIC1 in Fe transport was verified by the growth complementation of the Fe uptake deficient yeast strain fet3/fet4. Furthermore, Arabidopsis PIC1 overexpression lines (PIC1ox) accumulated about 2.5-times more Fe in chloroplasts than wild-type plants (Duy et al. 2011). Plants lacking PIC1 are characterized by a strong dwarf albino phenotype that is similar to Fe deficiency symptoms. In addition, pic1 knockout (ko) mutant plants display disrupted leaf mesophyll organization and a severely impaired chloroplast biogenesis, suggesting disturbed Fe homeostasis (Duy et al. 2007b). The function of PIC1 in chloroplast Fe uptake has been further supported by studies of PIC1 knockdown and overexpression lines in tobacco (Nicotiana tabacum) plants, which showed similar phenotypes to the Arabidopsis PIC1 mutants (Gong et al. 2015). In addition, Nt-PIC1 was as well able to complement growth of Fe deficient yeast fet3/fet4. For a previously proposed function of PIC1 in import of proteins, we refer to Lopez-Millan et al. (2016) and references therein.

A PIC1-NiCo translocon complex for Fe import. The interaction of PIC1 with the putative metal transport protein NiCo at the chloroplast IE membrane (Duy et al. 2011, Philippar 2018), further points to an essential role as permease in a multisubunit chloroplast Fe-translocon complex (Fig. 1). Plant NiCo proteins belong to the proton-motive-force driven nickel-, cobalt-transporter family described in bacteria (Eitinger et al. 2005). Thus, the proposed Fe^{2+} transport by a PIC1/NiCo complex in the IE membrane could represent the observed H^+-driven Fe^{2+} uptake by Shingles et al. (2002) in pea IE membrane vesicles. Action of both PIC1 and NiCo proteins in the same pathway is also reflected by the fact that transcript levels of the At-NiCo2 isoform are increased in PIC1ox mutant flowers (Duy et al. 2011). A point mutation of the NiCo ortholog in rice (named Os-ZN, for zebra-necrosis protein), although localized to thylakoids, caused a yellow-stripe, necrotic leaf phenotype (Li et al. 2010), which was light-dependent and related to ROS production. Arabidopsis PIC1 and NiCo proteins interact in yeast assays and pea orthologs co-migrate in native IE membrane complexes (Philippar 2018). Further, conditional knockdown mutants of Arabidopsis PIC1 and NiCo genes show similar albino phenotypes. Thus, a role of PIC1-NiCo in a translocon complex for Fe uptake across the IE of chloroplasts is likely (Duy et al. 2011). In developing leaves of B. napus, gene expression and protein amounts of PIC1 and NiCo are correlated (Pham et al. 2017). However, once leaves fully develop, expression of PIC1 decreases, while expression of NiCo remains rather stable (Müller et al. 2019), indicating that NiCo might have additional functions. Given that NiCo proteins contain specific metal binding domains (Eitinger et al. 2005), a possible molecular mechanism for PIC1-NiCo interaction in Arabidopsis IE membranes, could involve Fe^{2+} binding by NiCo and its subsequent transfer to the permease PIC1 (Fig. 1), similar to the mechanism of a novel class of prokaryotic ABC transporter complexes (see below). The still missing, soluble ATP-binding protein could be represented by At-ABCF1/GCN1 from the ABC protein subfamily F (Verrier et al. 2008), which encodes a dimeric NBD-subunit most likely targeted to chloroplasts and regulated in pic1 ko plants (Duy et al. 2007b). Since PIC1 represents a potential permease of an ancient, cyanobacterial origin, this hypothesis is further supported by the results of Kranzler et al. (2014) in Synechocystis. Here, the NBD subunit FutC also regulates the reduction-based Fe uptake of cyanobacterial cells via

the ABC transporter permease FutB. The transport mode for such a PIC1/NiCo ABC-complex would be a reduction-based, strategy I-like uptake of Fe2+ driven by the proton-motive force and membrane potential across the IE. The corresponding ferric chelate oxidoreductase would be FRO7 (see below and Fig. 1) as indicated by increased FRO6/FRO7 gene expression in PIC1ox plants (Duy et al. 2011).

Fe reduction at the IE by ferric chelate reductase enzymes. At-FRO7, a chloroplast-targeted ferric-chelate reductase (FCR)/oxidase was found to be fundamental for chloroplast Fe uptake under optimal iron nutrition (Jeong et al. 2008). In contrast to the root epidermis localized FRO2 enzyme, gene expression of FRO7 is not regulated by Fe deficiency (Mukherjee et al. 2006). Further, fro7 loss of function mutants do not show visible growth phenotypes under standard conditions and their phenotype under Fe deficiency can be rescued by Fe addition (Jeong et al. 2008). Thus, these results suggest the existence of redundant chloroplast Fe-uptake systems, which can complement the loss of FRO7. Nevertheless, Fe excess seems to be a negative regulator on both the expression and FCR activity in B. napus (Pham et al. 2017, Müller et al. 2019). In intact barley chloroplasts, FCR activity was induced by Fe deficiency under light, whereas it was repressed under dark conditions (Mikami et al. 2011). On chloroplast envelope fractions isolated from sugar beet, Solti et al. (2014) showed that similar to the strategy I Fe uptake in roots, an FCR enzyme was responsible for complexed FeIII-reduction and production of Fe2+ for Fe uptake. This transport was found to be light dependent, and FCR enzymatic activity was higher with NADPH than NADH. Since the reducing power of chloroplast FCR originates from NADPH produced in the light reaction of the photosynthetic apparatus, a direct connection between photosynthetic and Fe-uptake processes exists (Solti et al. 2014). Thus, for the proper function of the reduction-based Fe uptake of chloroplasts, the operation of the photosynthetic electron transport is essential. The biphasic kinetic of FCR and its modification under Fe-deficient conditions in addition indicate the existence of high and low affinity mechanisms for Fe reduction. In Arabidopsis most likely only one member of the FRO family —i.e. FRO7— is located in chloroplasts. Thus, these kinetics could be related to post-translational modifications, differential splicing or to the existence of a yet unknown Fe-reduction mechanism. Since the reduction-based Fe-uptake strategy of Synechocystis requires the contribution of the plasma membrane oxidoreductase ARTO, the Fe-uptake system of the chloroplasts also may be interpreted on the basis of the reductive Fe uptake of cyanobacteria. Thus, in angiosperm chloroplasts, ARTO would have been replaced by a chloroplast-targeted FRO, a FCR of eukaryotic origin.

MAR1/IREG3, a potential dually localized protein for Fe–NA uptake? For the Arabidopsis multiple antibiotic resistance 1 (MAR1/IREG3) protein, a localization to the chloroplast envelope and a role in cellular Fe homeostasis have been proposed (Conte et al. 2009, Conte and Lloyd 2010). First, MAR1/IREG3 gene expression is regulated by Fe deficiency, second MAR1/IREG3 overexpressing plants show leaf chlorosis that can be rescued by Fe addition (Conte et al. 2009, Zhang et al. 2018), and third this gene was found in two quantitative trait loci mapping for Arabidopsis seed mineral concentrations associated with seed Fe traits (Waters and Grusak 2008). Further, MAR1/IREG3 belongs to the IREG (iron regulated)/ferroportin transporter family, which mediate metal transport across the plasma membrane in the root stele (IREG1 for Fe loading to the vasculature) and tonoplast (IREG2 for Ni transport at Fe deficiency), respectively (Schaaf et al. 2006, Morrissey et al. 2009). Recent studies on MAR1/IREG3 suggest a possible dual localization in plastids and mitochondria, which was supported by intracellular GFP-targeting assays and the observation that gene expression of the mitochondrial ferric-chelate oxidoreductase FRO3 is increased in mar1/ireg3 ko lines (Zhang et al. 2018). Targeting peptide predictions also indicate a possible dual localization in chloroplasts and mitochondria (Aramemnon database) (Schwacke et al. 2003). Conte and co-

authors (Conte et al. 2009, Conte and Lloyd 2010) showed that MAR1/IREG3 can load aminoglycoside antibiotics into the chloroplast. In general, aminoglycosides are known to enter eukaryotic cells via polyamine inward transport systems (Van Bambeke et al. 2000). Since the polyamine-like NA in plants is of the most important natural chelators of Fe (for overview, see Curie et al. 2009), it is tempting to speculate that MAR1/IREG3 might transport NA or an Fe–NA complex into the chloroplast (Fig. 1; Conte et al. 2009, Conte and Lloyd 2010). The classification of MAR1/IREG3 into the ferroportin transporter family, however, argues against this idea, since ferroportins normally exchange Fe2+ with cations such as H+ or Na+.

YSL proteins for chloroplast Fe–NA export. Two transporters from the yellow stripe1-like family of oligopeptide transporters from Arabidopsis, YSL4 and YSL6, have been characterized as potential chloroplast Fe effluxers (Divol et al. 2013). Both of these genes are up-regulated in response to Fe excess and at least one of them, YSL6, was unequivocally localized to the chloroplast envelope (Fig. 1; Divol et al. 2013). However, if YSL6 integrates into the OE or IE, remains an open question, since neither YSL4 nor YSL6 contain a classical, N-terminal chloroplast targeting peptide, a feature that is more common for OE than for IE proteins. Phenotypical characterization of single and double knockout mutants showed that Fe accumulated in the chloroplasts and this occurred concomitantly with an increase in ferritin expression. Overexpression of YSL4 and YSL6 instead, caused sensitivity to Fe and a decrease of Fe in chloroplasts (Divol et al. 2013). In addition, the coordinated expression of these YSL genes with ferritin genes in embryos and senescent leaves prompted the suggestion for their physiological role in detoxifying Fe during plastid differentiation occurring in embryogenesis and senescence (Divol et al. 2013). The role of these transporters, however, remains controversial since due to proteomic data and GFP-targeting assays, YSL4 and YSL6 have also been described to insert into the tonoplast and ER membranes (Conte et al. 2013).

ABCI10, ABCI11 and ABCI12: Novel ABC-transporter subunits in Fe homeostasis. A chloroplast-localized, non-intrinsic ABC-transporter protein, NAP14 in Arabidopsis is similar to the FutC subunit of the FutABC Fe-transporter in cyanobacteria (Shimoni-Shor et al. 2010). At-NAP14 —also named ABCI11 according to ABC transporter nomenclature (Verrier et al. 2008)— encodes a non-membrane intrinsic, ATP-binding NBD-subunit of a prokaryotic-like ABC-transporter complex. In vivo GFP targeting by Shimoni-Shor et al. shows signals in the chloroplast stroma, own current results including immunoblot analysis point to an attachment of NAP14/ABCI11 to chloroplast-intrinsic membranes, most likely plastoglobuli and/or IE (L. Voith von Voithenberg, J. Park, R. Stübe, Y. Lee and K. Philippar, unpublished work). The Fe concentration in shoots of nap14 loss-of-function mutants is increased 18-fold over the wild-type plants. Moreover, this mutant showed damage to chloroplast structures, exhibited severe growth defects, strong chlorosis and a deregulation of Fe-homeostasis genes (Shimoni-Shor et al. 2010). Based on these findings, a function in regulating plastid Fe homeostasis and/or involvement in Fe/S cluster biogenesis similar to NAP7/SufC, a stroma localized NBD-NAP protein (Xu and Möller 2004, Balk and Schaedler 2014) is likely for NAP14/ABCI11. Potential membrane-intrinsic binding partners such as an ABC-transporter permease domain like FutB (Kranzler et al. 2014, Lau et al. 2016), which would anchor NAP14/ABCI to lipid layers, are still unknown (L. Voith von Voithenberg, J. Park, R. Stübe, Y. Lee and K. Philippar, unpublished work).

A direct role, as part of a plastid and prokaryotic-type metal ABC-transporter complex, is possible for the proteins ABCI10/NAP13 and ABCI12 as recently described (Philippar 2018, L. Voith von Voithenberg, J. Park, R. Stübe, Y. Lee and K. Philippar, unpublished work). Like ABCI11/NAP14, ABCI10/NAP13 represents a single, soluble NBD subunit of a multisubunit, prokaryotic-type ABC transporter complex. However, ABCI10/NAP13 shares slightly more similarity to NBD subunits of

non-classical energy coupling factor (ECF) ABC-transporter complexes (Rempel et al. 2018) than to these of the “normal” TMD/NBD dimers (Verrier et al. 2008) found in eukaryotes or to FutC in Gram-negative prokaryotes (Braun and Hantke 2011). Similar to ko mutants of ABCI11/NAP14 or PIC1 and NiCo, the loss-of-function of ABCI10/NAP13 in Arabidopsis causes severe albino and dwarfed plants with dramatically impaired chloroplast biogenesis. Again, shoots showed altered metal homeostasis including significantly increased Fe levels and changes in expression of Fe-transport and homeostasis genes (L. Voith von Voithenberg, J. Park, R. Stübe, Y. Lee and K. Philippar, unpublished work). ABCI10/NAP13, however, appears not to be associated with plastoglobuli but strongly bound only to IE membranes. Furthermore, the protein ABCI12 was identified as potential IE membrane-intrinsic interaction partner for ABCI10 by co-immunoprecipitation on chloroplast IE membranes as well as distinct staining patterns of co-transfected, fluorescence-tagged proteins (Philippar 2018, L. Voith von Voithenberg, J. Park, R. Stübe, Y. Lee and K. Philippar, unpublished work). ABCI-12 most likely inserts with five predicted α-helical transmembrane domains into the chloroplast IE membrane and represents the scaffold and energy-transducing subunit of a non-classical, prokaryotic-type ECF ABC-transporter complex (Bao et al. 2017, Rempel et al. 2018). In bacteria these ECF transporters —also known as energy-coupling factor transporters— are widely distributed and involved in the uptake of nickel and cobalt metal ions (group I CbiMNQO-type) or vitamin metabolites (group II EcfS/T/A-type). However, they have not been described in plants or any other eukaryotes. Therefore, we propose that ABCI-10 (as ATP-binding CbiO/EcfA subunit) and ABCI-12 (as the membrane-intrinsic energy-coupling subunit CbiQ/EcfT) in the IE (Fig. 1) are part of a novel chloroplast, metal ABC-transporter complex that was inherited by an ancient prokaryotic ancestor. The metal substrate for ABCI10/ABCI12 as well as the additional CbiM or EcfS subunits for substrate binding required for a functional membrane transfer of metal ions, however, remain to be identified.

In addition, proteomic studies and differential expression in chloroplast Fe-transport and homeostasis mutants, in combination with in silico predictions of plastid targeting demonstrated the presence of several ABC transporter subunits of still unknown function in or at the IE membrane. These include some “half-size” TMD/NBD proteins such as NAP8/ABCB28 (consisting of one transmembrane-domain permease and one soluble NBD domain, Verrier et al. 2008), as well as At-ABCF1/GCN1, a NBD-dimer (Duy et al. 2007b, Ferro et al. 2010, Gutierrez-Carbonell et al. 2014). The role of these proteins as interaction partners of metal translocon complexes and in plastid Fe homeostasis, however, deserves further study.

Plastids and Cellular Iron Homeostasis

As a sink for most of the cellular iron in mature leaves, chloroplasts are likely to be involved in sensing and regulation of Fe levels within the whole plant. In consequence, changes in the chloroplast Fe status may trigger different adaptation responses depending on the plant developmental stage (Vigani et al. 2013b). In spite of this important role, still little is known about processes governing Fe homeostasis and signaling in plastid organelles and about communication with other cellular compartments. In general, transcriptional profiling of plastid Fe-transport and -homeostasis mutants revealed that major changes occurred in genes related to intracellular metal transport and homeostasis. For both anterograde and retrograde signaling of the chloroplast Fe status, nitric oxide (NO) as well as redox and ROS signals are discussed (Dietz et al. 2016, Astier et al. 2018, Leister 2019). A comprehensive approach on the transcriptional response of Arabidopsis leaves to Fe deficiency (Rodriguez-Celma et al. 2013) led to the discovery of a set of genes encoding for small polypeptide proteins that are strongly up-regulated upon Fe shortage. Recently the ironman (IMA) subgroup of these proteins was nicely shown to regulate cellular Fe homeostasis,

most likely by Fe binding via a conserved aspartate-rich protein domain (Grillet et al. 2018). Over-expression of the cytosolic and nucleus-localized IMA1 lead to Fe toxicity in Arabidopsis as demonstrated by necrotic leaf spots, high Fe in leaves, stele and embryos as well as accumulation of numerous Fe-rich granules in chloroplasts (Grillet et al. 2018). Further, root FCR activity as well as expression of Fe-deficiency genes was up-regulated in IMA1ox plants. Silencing of all eight At-IMA genes in contrast lead to lethality of extremely chlorotic plants. In conclusion, Grillet et al. postulate that IMA proteins are regulatory peptides for control of cellular iron homeostasis. Although the At-IMA proteins studied so far are not in chloroplasts, plastid-targeted candidate proteins of the Fe deficiency transcriptome approach in leaves (Rodriguez-Celma et al. 2013), might be potential regulators and signal molecules for the chloroplast Fe status (Mokkapati and Schmidt 2018). Besides the well-studied mature chloroplast found in fully photosynthetically active leaves, non-green plastids in developing and senescent tissues ought to be included in future studies. Thus, to get a full picture, studies that on the one hand focus on specific organs/tissues such as mature leaves or seed development/germination (compare Fig. 3) and on the other hand integrate large data sets of transcriptomics, ionomics and proteomics are necessary to unravel organelle- and nucleus-driven signaling processes under disturbed Fe homeostasis.

Chloroplasts at Fe deficiency

Under Fe-deficient conditions, the Fe content of chloroplasts decreases significantly (Mikami et al. 2011), resulting in disturbed thylakoid biogenesis and decreased activity of the photosynthetic electron transport chain (Basa et al. 2014). Here, PSI is most sensitive against Fe deficiency and decreased root-to-shoot Fe translocation because of its high Fe4S4-cluster content (Andaluz et al. 2006, Yruela 2013, Basa et al. 2014). Nevertheless, under severe Fe deficiency, all thylakoid protein complexes are affected. The standard reduction-based, strategy I Fe uptake pathway at the IE membrane of mature chloroplasts under optimal Fe supply requires the operation of the photosynthetic electron transport chain (see before). However, since the latter is absent in developing proplastids, in etioplasts and in chloroplasts at Fe deficiency, additional mechanisms for Fe import are required. These may depend on different NADPH sources and/or transport proteins. For example reaction kinetics of the chloroplast FCR activity were changed under Fe deficiency, indicating that the reduction-based, pathway at the IE becomes less effective when iron is limiting (Solti et al. 2014). Accumulation of MAR1/IREG3 transcripts under Fe deficiency (Yang et al. 2010) suggests that an alternative Fe uptake pathway might occur via NA or NA-complexes (see above and Conte et al. 2009, Conte and Lloyd 2010). Nevertheless, the function of these potential NA–Fe transporters in the iron acquisition of non-photosynthetic and Fe-deficient plastids has not been tested. Furthermore, under Fe deficiency, the allocation of iron may also be altered among organelles, and Fe incorporation into components of the respiratory electron transport chain of mitochondria may be preferred; an effect that has already been observed under Fe starvation in the green microalgae Clamydomonas reinhardtii (Höhner et al. 2013) and in cucumber leaves (Vigani et al. 2018).

Fe release from plastids

Following the full development of the photosynthetic apparatus, and during senescence of photosynthetic tissues, cellular Fe distribution is reorganized to preserve iron for sink tissues. In senescent leaves, FER1 has a high expression probably as a consequence of the degradation of Fe-containing protein complexes and thus the increase of free Fe in degenerating chloroplasts. In Arabidopsis YSL4 and YSL6 (see above), were associated with the release of Fe during the dedifferentiation and senescence of chloroplasts as well as under Fe excess conditions to prevent

Fe overload (Fig. 1; Divol et al. 2013). The latter is underlined by up-regulation of YSL6 gene expression in PIC1 overexpressing lines that are characterized by increased Fe levels in chloroplasts (Duy et al. 2011). In ysl4/ysl6 double ko plants, however, the Fe content of generative tissues was not affected (Divol et al. 2013). Further, high expression of BnYsl4 was only found in ripening siliques, ripened seeds and cotyledons but not in early senescing leaves in B. napus (Müller et al. 2019).

In Arabidopsis, YSL6 is also implicated to be involved in the Fe release from plastids of germinating seedlings (Divol et al. 2013). In agreement, the expression of the ortholog YSL4 was high in ripening siliques, ripening seeds and cotyledons of seedlings in B. napus (Müller et al. 2019). Divol et al. (2013) also showed evidence for a crosstalk between the vacuolar and plastid Fe management as discussed below for vacuoles. Being transporters of the oligopeptide superfamily, YSL4 and YSL6 potentially transport oligopeptides like Fe-NA complexes (Pich et al. 1997, Solti et al. 2012, Müller et al. 2019), and thus the role of NA in plastid Fe transport may rather be in the remobilization of iron from chloroplasts or in non-green plastids of developing seeds and seedlings. A recent genome-wide study on YSL genes in wheat (Triticum aestivum) further supports specific roles for YSL proteins during grain development and under abiotic, biotic stress conditions (Kumar et al. 2019).

Impact of chloroplast Fe transport on cellular Fe homeostasis

Detailed phenotypic studies of PIC1 (Fe uptake) and ferritin (Fe storage) mutants in Arabidopsis lead to an interesting observation. On the one hand pic1 ko plastids show an accumulation of ferritin protein clusters, which most likely are loaded with iron (Duy et al. 2007b) (K. Philippar and G. Wanner, unpublished results). On the other hand, the phenotype of PIC1ox mutants clearly resembles that of ferritin loss-of-function plants under Fe excess, showing defects in flower and seed development that result in reduced seed yield and germination rates (Ravet et al. 2009a, Duy et al. 2011). Flowers of PIC1ox mutants contain more Fe, while other transition metals are unaffected, whereas seeds show a significant reduction in the concentration of this metal. Furthermore, PIC1 transcript levels are slightly up-regulated in ferritin knockouts (Ravet et al. 2009a). In summary, these findings point towards a close, reciprocal correlation between PIC1 and ferritin in photosynthetically active, mature leaf chloroplasts, which might be explained as follows. (i) In seed and meristem tissue, a plastid Fe uptake bypass pathway exists, which allows germination and slow growth of pic1 ko lines. Alternative Fe-uptake transporters in non-green plastids of embryo/seed development might be MAR1/IREG3, Mfl1 or ABC-I proteins (see above and Fig. 1). (ii) When leaves mature, chloroplasts become fully active in photosynthesis and therefore require a constant Fe import for maintenance of the photosynthetic apparatus. In this developmental phase Fe uptake mediated by PIC1 becomes dominant, and thus in pic1 ko plants, thylakoid biogenesis is interrupted due to the drastic block in Fe supply. This leads to the observed complete degradation of thylakoid membranes and release of the intrinsically bound Fe to the stroma. Since in mature leaf chloroplasts, ferritin protein levels to buffer this free Fe are low, ROS stress in the chloroplast stroma is likely. Under optimal Fe nutrition, no Fe microenvironment was detected to be associated to ferritin or NA-complexes (Solti et al. 2012, Müller et al. 2019). Moreover, increase in the Fe content of chloroplasts had a negative feedback on the Fe uptake by terminating the import at a certain plastid internal Fe concentration (Solti et al. 2012). Thus, in parallel, free Fe might accumulate in the cytosol, because again, an Fe-buffer or -sequestration system in mature leaf cells are not designated. Both, Fe-induced ROS signals and oxidative stress in the chloroplast and cytosol in turn might induce ferritin gene expression in the nucleus. (iii) In contrast, only chloroplast intrinsic levels of free Fe ions significantly increase in both, PIC1ox lines under Fe-sufficient and ferritin ko under Fe-excess growth conditions. In this situation, plants have to cope with oxidative stress in the plastid

stroma that leads to similar phenotypes concerning flower and, in particular, seed development. Increased transcripts for the potential, plastid Fe-NA chelate exporter YSL6 and the proposed Fe-NA/NA transporter MAR1/IREG3 (see above) in PIC1ox plants (Duy et al. 2011) point to a need to detoxify high free Fe levels in PIC1ox plastids.

Mitochondria and Iron

Mitochondria occupy a central place in the metabolic network of eukaryotes since they play a key role in cellular bioenergetic metabolism. Other than energy, plant mitochondria provide precursors for a number of essential biosynthetic processes. The metabolic complexity is mirrored by their complex architecture as revealed by 3D reconstruction of mitochondrial ultrastructure (Zick et al. 2009, Vigani et al. 2015). The general morphology of mitochondria is characterized by two membranes that divide the organelle into different compartments: the OM, the intermembrane space, the inner membrane (IM) and the matrix. Interestingly, mitochondria are also a focal point of Fe metabolism. Similar to what is observed for chloroplasts, the metallome of mitochondria isolated from Arabidopsis revealed that iron is the primary intrinsic micronutrient present (Nouet et al. 2011, Vigani and Hanikenne 2018). Mitochondria contain a large amount of metalloproteins that require iron (Vigani 2012). Considering the recent classification of Fe-requiring proteins (FeRE) (Vigani and Murgia 2018), as for chloroplasts almost all FeRE categories are represented in plant mitochondria (Fig. 2). The majority of Fe is required for proteins belonging to the electron transport chain (Vigani et al. 2009). Additionally, Fe is required by enzymes involved in molybdenum (Mo) cofactor biosynthesis (Bittner 2014, Vigani et al. 2017), ROS scavenging by peroxidase, DNA metabolism and dioxygenases. In the mitochondrial FeREs, iron plays its biological function as enzyme cofactor like Fe-heme groups, Fe–S clusters and di-iron centers (Vigani and Hanikenne 2018). Specific Fe–S cluster assembly machineries operate in mitochondria as well as in some steps useful for the synthesis of heme (see below). Generally, in plant cells, mitochondria seem to play a more central role for the synthesis of cellular Fe–S clusters, because they provide glutathione persulfide as sulfur suorce for cytosolic Fe–S cluster assembly. A mitochondrial transporter mediating the efflux of these precursors has been identified in yeast, plant and mammals: the ATP-binding cassette transporter of mitochondria (ATM). Proteins of this ATM-subfamily belong to the plant ABC-transporter group B (Verrier et al. 2008, Bernard et al. 2009). In Arabidopsis, it has been shown that ATM3 is involved in the export of glutathione polysulfide from mitochondria, which provides the persulfide required for both Fe–S cluster assembly and Mo cofactor biosynthesis in the cytosol (Schaedler et al. 2014). Thus, mitochondria organelles are crucial for cellular metal-homeostasis in general.

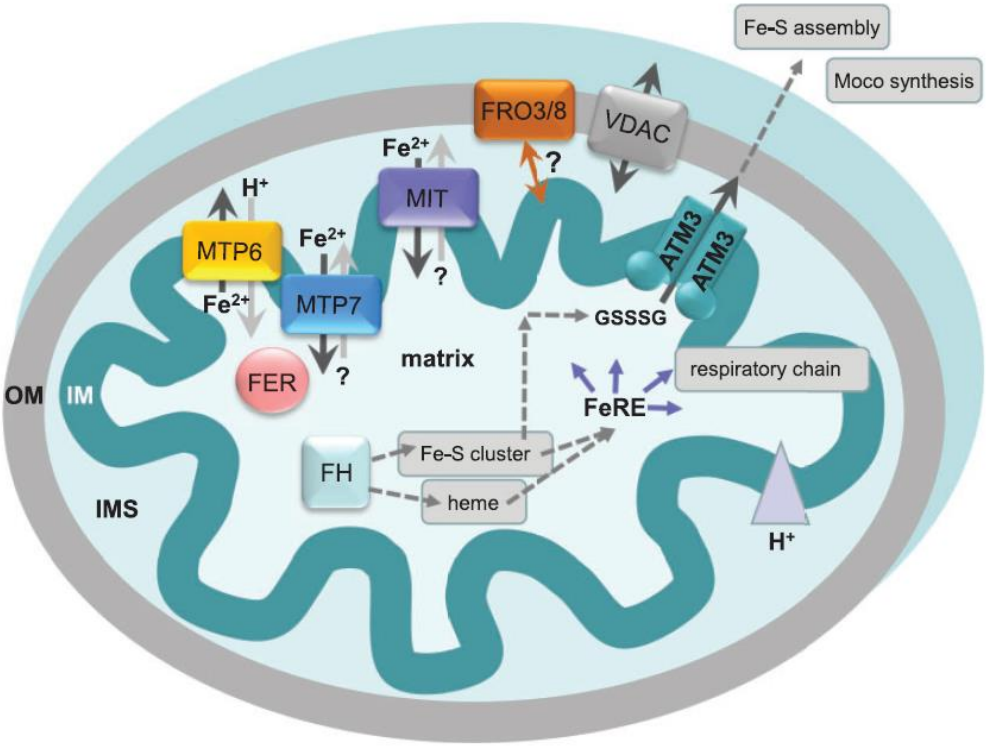

Fig. 2

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Iron transport and homeostasis in mitochondria. Ion and metabolite transport across the outer membrane of mitochondria (OM) is supposed to occur via the general ß-barrel porin VDAC. Iron transport over the strongly invaginated inner membrane (IM) is assumed to take place in the form of Fe2+. Thus, Fe-transport mechanisms at the OM, IM of mitochondria appear to be similar to the pathways for chloroplast envelope membranes (see Fig. 1). For Fe reduction, the ferric chelate reductases FRO3 and FRO8 have been implicated with a function in mitochondria. However, it is still unclear whether FRO3 or FRO8 are at the OM or IM and thus unknown if cytosolic or intermembrane space (IMS) FeIII-chelates are substrates for mitochondrial FRO enzymes. For uptake of Fe2+ the MIT protein, belonging to the mitochondrial carrier family (MCF), is the candidate of choice. However, since the generalized transport reaction for carriers of the MC family is an antiport mechanism (compare TCDB at http://www.tcdb.org) the counterion for Fe2+ transport by MIT is still unknown. In addition, the proteins MTP6 and MTP7, which belong to the Fe/Zn CDF family (cation diffusion facilitator), are described to shuttle Fe across the IM. In exchange with H+, MTP6 most likely exports Fe2+ at Fe overload from the matrix. At cytosolic Fe excess, however, MTP7 is believed to deliver Fe to mitochondrial ferritin (FER) for storage and prevention of ROS stress (compare Fig. 1 for chloroplast FER). At normal Fe supply in the mitochondrial matrix, frataxin (FH) supplies iron for Fe–S cluster and heme biosynthesis. Further, the assembled Fe-requiring proteins (FeR) are involved in manifold metabolic functions as described in the text. Electron transport by the respiratory chain, which also generates a strong H+ gradient (grey triangle) across the IM, here represents the most prominent pathway. The sulfur source for cytosolic Fe–S cluster assembly and

molybdenum cofactor (MoCo) synthesis is also provided by mitochondria via export of glutathione polysulfide (GSSSG) by the IM-resident ABC transporter ATM3. For all membrane proteins, the predicted number of α-helical transmembrane domains (α-TMD, Aramemnon database) (Schwacke et al. 2003) as well as the designated families according to TCBD (Saier et al. 2006) are as follows: ATM3 (2× 6 α-TMD-NBD, ABCB subfamily ABC transporter, TC 3.A.1_ATM-type); FRO3, FRO8 (11 α-TMD); MIT (6 α-TMD, mitochondrial carrier family, TC 2.A.29_MIT-type); MTP6, MTP7 (6 α-TMD, other cation carrier group-CDF family, TC 2.A.4_Fe/Zn CDF-type).
Fig. 2

Mitochondrial Fe homeostasis

Since iron is essential for high number of mitochondrial FeRE, Fe homeostasis in mitochondria must be tightly regulated, which requires the function of specific proteins. In this context, particular attention has been paid on the role of ferritin and frataxin (FH), which are implicated in Fe storage and control of Fe homeostasis in the mitochondrial matrix (Corsi et al. 2002) as well as in nutrient homeostasis of the plant cell (Murgia and Vigani 2015). Despite the fact that the four ferritin proteins (FER1–4) identified in Arabidopsis are known to be localized to chloroplasts (see above), some evidence suggests that FER also are intrinsic to plant mitochondria (Fig. 2) (Zancani et al. 2004, Tarantino et al. 2010). It has been demonstrated that At-FER4 might be involved in mitochondrial Fe homeostasis (Tarantino et al. 2010) and accordingly, a native gel electrophoresis followed by Prussian blue staining revealed that ferritin is a functional Fe-storage protein in mitochondrial fraction under Fe excess condition in cucumber plants (Vigani et al. 2013a).

FH is a conserved mitochondrial protein found in several eukaryotic organisms including plants (Busi et al. 2004). The Arabidopsis FH loss-of-function (atfh) mutants displayed an embryo lethal phenotype, indicating that At-FH is essential (Vazzola et al. 2007). Furthermore, atfh mutants showed reduced activity of two Fe–S cluster containing enzymes, mitochondrial aconitase and succinate dehydrogenase, suggesting that FH plays a role in Fe–S cluster biogenesis (Fig. 2). Overall, the synthesis and assembly of Fe–S clusters in organelles like mitochondria or chloroplasts involve the action of scaffold proteins interacting with iron- and sulfur-delivery proteins (Balk and Schaedler 2014). It has been suggested that FH promotes the interaction with the ISU scaffold proteins favoring sulfur transfer reactions and the subsequent binding with iron (Couturier et al. 2013). Indeed, it was shown that At-FH plays an instrumental role in Fe–S cluster biogenesis in plant mitochondria (Turowski et al. 2012). Here At-FH interacts with a cysteine desulfurase (At-NFS1), which supplies sulfur to the Fe–S cluster machinery and modulates its kinetic properties. In addition, Buchensky et al. (2017) showed that in Zea mays, the two frataxin isoforms (Zm-FH1 and Zm-FH2) have a dual localization in mitochondria and chloroplasts (see Fig. 1). Since FH is the primary iron donor of the mitochondrial Fe–S cluster biosynthesis, it is suggested to have the same function for the chloroplast Fe–S cluster machinery (Turowski et al. 2015, Gomez-Casati et al. 2018).

Other than its role in the Fe–S cluster assembly in the mitochondrial matrix, FH also seems to participate in heme biosynthesis (Fig. 2). Heme is a highly stable structure where Fe is coordinated in a tetrapyrrole ring. Although in plants heme biosynthesis is known to be localized in plastids, recent reports have indicated that the insertion of Fe into the tetrapyrrole ring by ferrochelatase (FC), may also take place in mitochondria (Tanaka and Tanaka 2007, Mochizuki et al. 2010). Further, atfh mutants displayed a decreased heme content as well as a down-regulation of several genes involved in heme biosynthesis (Maliandi et al. 2011). In humans, it has been demonstrated that FH delivers Fe to FC for heme biosynthesis in mitochondria (Yoon and Cowan 2004). In plants, the final step of the heme biosynthesis pathway, which inserts ferrous iron into protoporphyrin IX (Espinas

et al. 2016) is mediated by the two isoforms FC1 and FC2. Despite the fact that plant FC seems to be primarily located in plastids (Roper and Smith 1997, Masuda et al. 2003), some studies reported that FC1 could also be located in mitochondria (Chow et al. 1997, Hey et al. 2016). Recently, it has been shown that in the presence of both iron and protoporphyrin IX, FH itself seems to exhibit FC activity by catalyzing the formation of heme in vitro. In addition, FC-activity increased when FH was combined with proteins of the Fe–S cluster assembly machinery. Therefore, it has been suggested that FH might act as Fe donor in the final step of heme synthesis in plant mitochondria (Armas et al. 2019).

Thus, in summary two of the central intracellular proteins for Fe homeostasis —ferritin and frataxin— both appear to share a dual localization in the stroma of chloroplasts and the matrix of mitochondria, being involved in Fe storage as well as Fe–S cluster and heme cofactor biosynthesis in both organelles. A possible interaction between FH and ferritin in mitochondria has been found in Arabidopsis plants (Murgia and Vigani 2015). Here, a comparative analysis of the leaf ionome profile of fer4, atfh single and fer/atfh double ko mutants revealed that changes in the mineral fingerprint of the double mutant involve at least Na, K, Fe and Mo. In conclusion, these results suggest a contribution of both At-FH and At-FER4 in the cellular ionome profile. In particular, the alterations of Mo-content in the double mutant with respect to single fer4 mutants highlight the link of Fe–Mo crosstalk in plant mitochondria (Murgia and Vigani 2015, Vigani et al. 2017).

Iron Transport in Mitochondria

Plant mitochondrial Fe-import proteins belong to the mitochondrial carrier family (MCF) and localize to the mitochondrial IM (Kunji and Robinson 2006, Bashir et al. 2011a, Bashir et al. 2011b). Mitochondrial Fe transporters (named mitoferrins) have been described in several eukaryotic organisms, including yeast and humans (Foury and Roganti 2002, Paradkar et al. 2009). In plants, first evidence for a mitoferrin-like gene (Mfl1) was found in Arabidopsis (Tarantino et al. 2011). However, Tarantino et al. showed that most likely At-Mfl1 is involved in Fe trafficking in chloroplasts (Fig. 1) (Lopez-Millan et al. 2016). Ion and metabolite transport across the OM of mitochondria is supposed to occur via the general ß-barrel porin VDAC (voltage-dependent anion channel) (Colombini 2012).

Fe uptake by MIT proteins. The first identification and characterization of a true mitochondrial iron transporter (MIT, Fig. 2) of the MCF family was obtained with rice (Oryza sativa) (Bashir et al. 2011b). MIT is an essential gene for rice and MIT knockdown mutants (mit2) exhibit a slow growth phenotype and reduced chlorophyll content. Additionally, mit2 mutants displayed a decreased Fe content in mitochondria and the expression of MIT was induced under Fe deficiency, indicating that MIT is involved in Fe loading to mitochondria under low iron availability. Accordingly, the role of MIT transporters in delivering Fe to mitochondria under sufficient Fe supply and Fe deficiency has been observed in cucumber plants (Migocka et al. 2018). The types of Fe species in the various subcellular compartments of cells are not yet clear; however, it is accepted that the form of iron transported across the mitochondrial inner membrane is Fe^{2+} (Jain and Connolly 2013).

Fe reduction by ferric-chelate reductase enzymes. As well known for the root plasma membrane and most likely for chloroplast Fe uptake (see before), it has been postulated that also mitochondrial Fe uptake involves an Fe-reductase activity. Such activity might be mediated by metalloreductase enzymes belonging to the FRO family (Jeong and Connolly 2009). Although any direct evidence for an involvement of reductase proteins in mitochondrial Fe metabolism is lacking, some members of the FRO family, such as FRO3 and FRO8, are predicted to localize to mitochondria (Fig. 2) (Heazlewood et al. 2004, Jeong and Connolly 2009, Jain and Connolly 2013). Whereas FRO3 transcript content is high in seedlings, expression of FRO8 is restricted to shoots during senescence

(Mukherjee et al. 2006, Jeong and Connolly 2009), suggesting that both enzymes may be involved in Fe reduction at different stages of development. Recently, it was shown that At-FRO3 most likely targets to the OM of mitochondria and thus might use cytosolic NADH for reduction of FeIII (Connolly et al. 2018). However, in rice, the two identified members of the FRO family do not seem to localize to the mitochondria (Victoria et al. 2012, Jain and Connolly 2013), and therefore the exact role of FRO3 and FRO8 still remains to be clarified.

Fe-transport by other carriers. In addition to the MCF family, low-affinity transport systems may be involved in the increased accumulation of iron in mitochondria (Muhlenhoff et al. 2003). The cation diffusion facilitator (CDF) family comprises divalent heavy metal cation transporters that are ubiquitous in all kingdoms of life. Members of this family have been classified into three phylogenetically different subgroups —Zn-CDF, Fe/Zn-CDF and Mn-CDF— based on their confirmed or putative metal specificity (Montanini et al. 2007, Gustin et al. 2011). Proteins of plant Fe/Zn-CDFs are designated metal tolerance proteins 6 (MTP6) and 7 (MTP7), respectively (Gustin et al. 2011). The role of MTP6 and MTP7 proteins has been recently characterized in cucumber plants. The zinc transport function of MTP6 has not yet been confirmed, but evidence indicates that MTP6 is involved in Mn/Fe efflux from mitochondria (Fig. 2) by catalyzing the export of Fe2+ and Mn2+ from mitochondria via an Fe2+(Mn2+)/H+ antiport (Migocka et al. 2019). Further, it has been shown that Cs-MTP7 is a mitochondrial transporter involved in Fe-accumulation in mitochondria (Migocka et al. 2018). Migocka et al. demonstrate that cucumber MTP7 function is tightly linked to ferritin and mitochondrial Fe-storage, suggesting that mitochondria might be able to accumulate and store iron under Fe excess. Therefore, based on the effect of iron on the expression of both genes MTP6 and MTP7, it has been hypothesized that on the one hand expression of Cs-MTP7 is up-regulated by elevated Fe to increase the mitochondrial content for Fe-storage. On the other hand, Cs-MTP6 expression is increased both by elevated iron to remove excess Fe and reduce toxicity, and by Fe-deficiency to release iron stored in mitochondria in order to meet cellular demands (Migocka et al. 2019).

According to latest reports also the potentially dually localized MAR1/IREG3 protein, belonging to a different subgroup of carriers (Aramemnon database; Schwacke et al. 2003), might contribute to mitochondrial Fe transport (see above for chloroplast Fe transport; Zhang et al. 2018).

Mitochondrial Fe transport and cellular iron homeostasis

Evidence about impaired mitochondrial Fe uptake on plant Fe homeostasis and metabolism so far has been provided only in rice. Indeed, the mit2 ko mutation leads to wide alterations of metabolism as well as a mis-localization of Fe in the cell (Bashir et al. 2011a, Bashir et al. 2011b, Vigani et al. 2016). An upregulation of the expression of the vacuolar iron transporter1 gene (VIT1, see below) in mit2, suggested that excess cytosolic iron may be directed toward vacuoles. As expected, the loss of mitochondrial Fe uptake in rice affects heme and Fe–S cluster synthesis (Vigani et al. 2016). At the root level, mit2 mutation affects metabolism by inducing alternative respiratory pathways and related changes in the level of amino acids of the aspartate family, while in shoots ornithine and ROS accumulation is induced (Vigani et al. 2016). Whether such changes are due to the impaired activity of FeRE enzymes in the cell or to an as yet unknown retrograde signaling pathway still remains to be elucidated. Furthermore, the role of mitochondria in the regulation of Fe deficiency induced responses has been recently highlighted in the Arabidopsis abscisic acid-hypersensitive mutant (ahg2-1), which is known to be defective in mitochondrial mRNA regulation. ahg2-1 displayed an increased expression of some iron deficiency response genes as well as the expression of a novel gene encoding a short polypeptide named FEP1, which has been shown to play a role in the induction of Fe-uptake genes in Arabidopsis (Hirayama et al. 2018).

Vacuoles and Iron

Vacuoles occupy over 90% of the cell volume in most plant cell types and therefore constitute a compartment of choice for ion storage. However, in a typical mesophyll cell, most of the iron is localized in chloroplasts. In higher plants, therefore Fe excess triggers increased Fe storage in FER, which are localized in plastids and mitochondria, rather than alternative storage in vacuoles (Briat et al. 2010a, Briat et al. 2010b).

Iron storage in seed vacuoles

The role of vacuoles in Fe storage was elucidated while investigating the mechanisms of seed iron storage in Arabidopsis. Disruption of At-VIT1, a vacuolar Fe transporter expressed mostly in seeds, was found to disrupt Fe distribution in seeds (Kim et al. 2006). Whereas in wild-type seeds, iron is concentrated in cells surrounding the provasculature of the embryo, in vit1 loss-of-function mutants, iron is found in the subepidermal cell layer. Moreover, two vacuolar metal transporters, NRAMP3 and NRAMP4 (natural resistance-associated macrophage protein), were shown to be necessary for the retrieval of Fe stores during germination. Further studies, using techniques to map iron with subcellular resolution, confirmed that Fe co-localized with globoids within seed storage vacuoles (Lanquar et al. 2005, Roschzttardtz et al. 2009, Mary et al. 2015). Thus, in conclusion, VIT1, NRAMP3 and NRAMP4 function as a Fe storage and remobilization module in endodermal cells of embryos (Fig. 3). Mutant seedlings deficient in these transporters display strong chlorosis and developmental arrest when germinated in Fe-free medium, indicating the predominance of vacuolar Fe stores in seeds. Based on the analysis of ferritin mutants, Ravet et al. (2009b) estimated that no more than 5% of Arabidopsis seed iron is associated to ferritin in plastids. In the absence of VIT1, the CDF transporter MTP8, responsible for the storage of Mn in subepidermal cell vacuoles, also loads Fe together with Mn into vacuoles of this cell type (Chu et al. 2017, Eroglu et al. 2017). Conversely, in the absence of MTP8, Mn is stored in endodermal vacuoles together with Fe (Chu et al. 2017, Eroglu et al. 2017). Both VIT1 and MTP8 can transport Fe and Mn but with different affinities: VIT1 has higher affinity for Fe and MTP8 higher affinity for Mn. Interestingly, NRAMP3 and NRAMP4 are also able to export both Fe and Mn from the vacuole (Lanquar et al. 2010, Thomine et al. 2003). During seed development, iron first transits into other cellular compartments resembling the endoplasmic reticulum before being deposited in vacuoles at later stages of embryogenesis (Ibeas et al. 2017). In Arabidopsis, the role of the endodermal vacuole in seed Fe storage is thus firmly established (Fig. 3), while it is still a matter of debate in other dicotyledonous seeds. Very recent work indicates that endodermal Fe storage could be restricted to dicot Brassicaceae (Ibeas et al. 2018). In monocotyledonous species, Fe is stored in the aleurone layer surrounding the endosperm and in the embryo. The subcellular localization of iron in embryo cells is not known. In the aleurone cell layer, Fe has been localized to globoids corresponding to the vacuolar compartment (Lott and Spitzer 1980). Recently, ectopic expression of a wheat VIT ortholog in wheat endosperm was shown to increase available wheat grain Fe, indicating that increasing vacuolar Fe stores is a valid strategy for biofortification (Connorton et al. 2017).

Vacuolar Fe storage in vegetative tissues

Besides seeds, it is likely that vacuoles are also involved in Fe storage at vegetative stages. Whereas, At-VIT1expression pattern is restricted to seed development, several VIT1 homologs, named VTLs or Nodulin-like, are expressed at vegetative stages and up-regulated in roots when Fe availability increases (Gollhofer et al. 2011). Their expression is controlled by one of the central regulators of Fe homeostasis ILR3/bHLH105 (Rampey et al. 2006). Some members of this family were demonstrated to encode bona fide vacuolar Fe transporters (Gollhofer et al. 2011). Moreover, AtNRAMP3 and AtNRAMP4 are also expressed in vegetative organs and up-regulated under Fe

deficient conditions (Lanquar et al. 2010). So far, only their role in Mn remobilization has been documented in mesophyll cells. They may play a role in Fe remobilization from vacuoles in other organs such as roots for example. Os-VIT1 and Os-VIT2 have been shown to control Fe and Zn storage in leaves, indicating that in rice mesophyll vacuoles are involved in Fe storage (Zhang et al. 2012). In contrast, in Arabidopsis, mesophyll vacuoles contain only a small proportion of cellular Fe. Iron loading in the vacuole is also important for determination of the petal color. In tulip (Tulipa gesneriana) and more recently in cornflower (Centorea cyanus), the blue pigment accumulated in petal cell vacuoles is formed by a complex between anthocyanins and ferric Fe. Here, expression of a functional VIT1 transporter is important to confer the dark blue color found at the bottom of the petal in some tulip varieties as well as the blue color to cornflower petals (Momonoi et al. 2009, Yoshida and Negishi 2013). Interestingly, the part of the tulip petal that is not dark blue expresses higher levels of ferritin than the dark blue part. Concomitant high expression of the vacuolar iron transporter and decreased expression of plastid ferritin is thus required to achieve dark blue color (Shoji et al. 2010).

Iron speciation in vacuoles

Even though vacuoles are not metabolically active, it is unlikely that iron is present in a free ionic form in the vacuolar lumen. To which ligands Fe is bound in vacuoles has not been unequivocally determined. In seeds, the strong co-localization of iron with globoids suggests that FeIII is bound to phytate, as shown for Mn (Fig. 3) (Bruch et al. 2015). In seedlings, Fe was also shown to co-localize with phosphorus when phosphate is available in the medium (Hirsch et al. 2006). Iron is thought to be stored as ferric (Fe^{3+}) iron in vacuoles. The speciation of iron and its redox status in vacuoles are being formally demonstrated, using techniques such as X-ray absorption spectroscopy (Chay et al. 2018). As NRAMPs are divalent metal transporters, this implies that FeIII is reduced to Fe^{2+} prior to its export from the vacuole. This could be achieved by a tonoplast bound FCR. However, none of the members of the FRO family were identified in the tonoplast membrane (Jain et al. 2014). Recent results indicate that influx of ascorbate into the vacuole participates in FeIII reduction in the vacuole during its remobilization (Chay et al. 2018). In addition, it is possible that coumarins, similar to those that are secreted in the rhizosphere and have been shown to be capable of Fe reduction (Rajniak et al. 2018) are loaded into the vacuolar lumen by vacuolar ABC transporters. NA could also play a role as ligand for Fe in vacuoles. Since ZIF1, a NA-transporter in the tonoplast, is up-regulated under Fe deficiency (Haydon et al. 2012), loading of NA could help making vacuolar iron more available. Two putative transporters of NA–Fe, YSL4 and YSL6 (see before), have also been identified in the tonoplast proteome (Jaquinod et al. 2007, Conte et al. 2013). However, their localization in this compartment was questioned by immunolocalization experiments showing that both proteins reside in the chloroplast envelope (Divol et al. 2013).

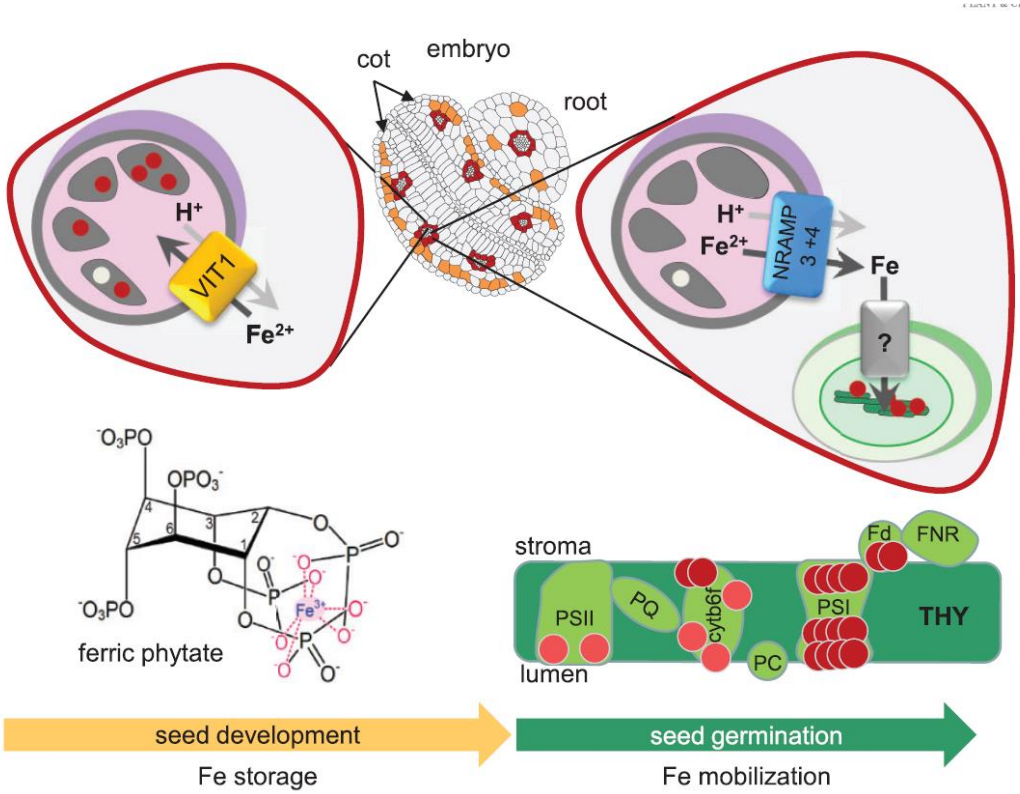

Fig. 3 Iron storage and remobilization in Arabidopsis seed vacuoles. (Left) During embryo development in seeds, the transporter VIT1 drives Fe (red dots) for storage into the vacuoles (pink) of endodermal/perivascular cells (red) in cotyledons and roots. VIT1 most likely transports Fe2+ by an antiport with H+, driven by the proton gradient across the tonoplast. In these storage vacuoles, Fe is associated to phytate globoids (dark grey) and thought to be stored as ferric phytate. (Right) During seed germination and early seedling development, the vacuolar Fe is remobilized by export via NRAMP3, NRAMP4 (H+ symport mechanism) and used preferentially to provide Fe to chloroplasts, where it used for photosynthetic electron transport in thylakoid (THY) membranes. Besides the Fe-containing photosystem II (PSII), the cytochrome b6f complex, and ferredoxin (Fd), here PSI with three Fe4–S4 clusters represents the richest Fe system. The Fe-import pathway into these developing chloroplasts, however, is still unknown. For Arabidopsis VIT1 and NRAMP3, NRAMP4, the predicted number of α-helical transmembrane domains (α-TMD, Aramemnon database) (Schwacke et al. 2003) as well as the designated families according to TCBD (Saier et al. 2006) are: VIT1 (5 α-TMD, other carrier groups-VIT family, TC 2.A.89_VIT-type); NRAMP3, NRMAP4 (12 α-TMD, other carrier groups-NRAMP family, TC 2.A.55_NRAMP-type).

A tight connection between vacuolar and plastid Fe pools

Ravet et al. (2009b) provided evidence for cross-talk between vacuolar and plastid Fe pools: the accumulation of the seed plastid ferritin FER2 is decreased in VIT1 overexpressing plants with increased Fe storage in vacuoles or in nramp3nramp4 mutants, in which vacuolar Fe stores cannot be retrieved. Divol et al. (2013) also showed that in the absence of YSL4 and YSL6 — putative NA or Fe–NA transporters of the chloroplast envelope (see Fig. 1)— the expression of the vacuolar transporters AtNRAMP3 and AtNRAMP4 is strongly decreased. Transcriptomic analyses of the nramp3/4 double ko mutant during germination indicated that genes encoding plastid Fe-

containing proteins are specifically down-regulated when vacuolar iron cannot be remobilized from the vacuole, even in the presence of iron in the external medium (Bastow et al. 2018). Further, the nramp3/4 ko mutant does not contain FER2, the seed stable ferritin isoform, which suggests a decrease of Fe in the plastid (Ravet et al. 2009b). Together, these results suggest that in seed tissue the vacuolar Fe is a privileged source of Fe for plastids (Fig. 3), and that Fe fluxes across the plastid envelope and the tonoplast are tightly coordinated. However, as previously discussed by Divol et al. (2013), the expression of these genes may not occur in the same cells and therefore the cross-talk between the vacuolar NRAMP3/4 and the plastid YSL4/6 must involve Fe sensing in the plastid and signaling to adjust Fe release from the vacuole.

The role of vacuoles in metal sequestration under Fe starvation

In addition to their role in Fe storage, root vacuoles play a very important role during Fe deficiency. In addition to inducing the expression of the Fe import machinery in roots, Fe deficiency also triggers increased expression of several vacuolar transporters for other metals that are inadvertently imported in root cells by IRT1 together with Fe. These tonoplast metal transporters are MTP3, IREG2, HMA3 and MTP8, which sequestrate Zn, Ni, Cd and Mn in root vacuoles, respectively (Arrivault et al. 2006, Schaaf et al. 2006, Morel et al. 2009, Eroglu et al. 2016). Root vacuoles thus play a protective role as a firewall capturing these potentially toxic cations and preventing them from reaching the shoots (Thomine and Vert 2013).

Outlook and Perspectives

Identification of molecular components for iron transport and homeostasis in combination with the physiological characterization of corresponding mutants in model plants in the last decade lead to a considerable insight into the mechanisms and pathways for intracellular iron distribution in land plants. Furthermore, subcellular resolution was achieved by refined techniques for metal detection, ultrastructural analysis and transport studies on isolated organelles. To complete the picture, several challenges remain for the future. For all proteins involved, a clear and unequivocal subcellular localization is required, in particular because evidence is accumulating for possible dual functions in chloroplasts and mitochondria. For these endosymbiotic organelles, comparison to prokaryotic pathways can definitely be helpful. Research on plant membrane metal transport proteins still faces the contest to develop straightforward functional assays to clarify substrates and transport modes. Further, in higher plants studies have to consider different developmental stages and organs as well as diversities and similarities between dicots and monocots. Although quite fundamental, still very little is known about signaling pathways, corresponding to the metal status of organelles. Besides the general suspects, such as ROS or NO signals, the integration of small, IMA-like metal-binding proteins as endogenous reporter and signaling proteins is very promising. In addition, the development of artificial intracellular sensors such as redox-sensitive GFP variants will contribute to track signaling pathways. Furthermore, the future integration of omics data by global in silico analysis should help to unravel the impact of iron nutrition and interactions in plants.

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