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REVIEW PAPER

Iron in leaves: chemical forms, signalling, and in-cell distribution

Máté Sági-Kazár[1,2], Katalin Solymosi[3], and Ádám Solti[1,*]

[1] Department of Plant Physiology and Molecular Plant Biology, Institute of Biology, ELTE Eötvös Loránd University, Pázmány Péter sétány 1/C, Budapest, H-1117, Hungary
[2] Doctoral School of Biology, Institute of Biology, ELTE Eötvös Loránd University, Pázmány Péter sétány 1/C, Budapest, H-1117, Hungary
[3] Department of Plant Anatomy, Institute of Biology, ELTE Eötvös Loránd University, Pázmány Péter sétány 1/C, Budapest, H-1117, Hungary

* Correspondence: adam.solti@ttk.elte.hu

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Abstract

Iron (Fe) is an essential transition metal. Based on its redox-active nature under biological conditions, various Fe compounds serve as cofactors in redox enzymes. In plants, the photosynthetic machinery has the highest demand for Fe. In consequence, the delivery and incorporation of Fe into cofactors of the photosynthetic apparatus is the focus of Fe metabolism in leaves. Disturbance of foliar Fe homeostasis leads to impaired biosynthesis of chlorophylls and composition of the photosynthetic machinery. Nevertheless, mitochondrial function also has a significant demand for Fe. The proper incorporation of Fe into proteins and cofactors as well as a balanced intracellular Fe status in leaf cells require the ability to sense Fe, but may also rely on indirect signals that report on the physiological processes connected to Fe homeostasis. Although multiple pieces of information have been gained on Fe signalling in roots, the regulation of Fe status in leaves has not yet been clarified in detail. In this review, we give an overview on current knowledge of foliar Fe homeostasis, from the chemical forms to the allocation and sensing of Fe in leaves.

Keywords: Chloroplast, citrate, DNA methylation, glutaredoxin, glutathione, hemerythrin, histone modification, iron–sulfur cluster, mesophyll, mitochondrion.

Introduction

Iron (Fe) is one of the most important transition metals in plants required for essential cell functions. The known oxidation states of Fe in biological environments range from Fe(II) to Fe(IV) (heme), the chemistry of which is multiplied by accessible spin states (for review, see Chen and Browne, 2018). In biological systems, Fe-dependent redox reactions occur in a broad electrochemical potential. In consequence, Fe cofactor-containing redox enzymes are common. In plants, the photosynthetic apparatus has a particularly high need for these Fe cofactors. Thus, foliar Fe homeostasis plays a crucial role in the physiological status of the plant, since autotrophy relies on the availability of Fe (Box 1).

Abbreviations: Asc, Ascorbate;Cit, citrate; DNIC, dinitrosyl Fe complexes;GSH, glutathione; Mal, malate; MNIC, mononitrosyl Fe complexes; NA, nicotianamine; ROS, reactive oxygen species.

Box 1. Iron transport and homeostasis of photosynthetically active cells in leaves support the need for iron in chloroplasts.

Higher plants primarily take up Fe by their roots, but due to the high demand for Fe to build up and operate the photosynthetic apparatus in the chloroplasts of photosynthetically active tissues, the largest proportion of Fe is translocated to the leaves. The high demand for Fe in the photosynthetic apparatus is supported by the Fe-carboxylates translocated in the xylem sap and taken up by leaf cells through the reduction-based Fe transport strategy. Once Fe is taken up by the cells, it becomes part of the labile Fe pool of the cytoplasm. Characterization of this pool has multiple technical difficulties since the concentration of Fe remains below the threshold of Fe speciation techniques and isolation of Fe with intact micro surrounding has not been resolved either. Although Fe has a primary importance in photosynthesis, mitochondrial function also requires a significant amount of Fe. In comparison to roots, where Fe management is dominated by the acquisition of Fe and its translocation towards sink tissues, Fe homeostasis of leaves is for the most part dependent on the incorporation of Fe into cofactors of redox enzymes. Nevertheless, the direction of the intracellular Fe allocation in leaves is also dependent on the developmental programme. During leaf development, the biosynthesis of the photosynthetic apparatus dominates. Information on Fe management in leaf primordia before the intensive development of chloroplasts and their photosynthetic apparatus is highly limited. Fully developed mature leaves support the carbon, nitrogen, and sulfur autotrophy of the plant. Sooner or later, leaves turn to senescence and become a source of organic and inorganic material. During senescence, Fe is liberated from the photosynthetic apparatus, leading to an intracellular reallocation of Fe and a remobilization towards new sink tissues. Since the vegetative parts of plants are also important in human nutrition, understanding Fe homeostasis and its regulation in leaves could also lead to improved plant breeding and pave the way for high-precision agriculture techniques. Moreover, understanding the mechanisms of Fe remobilization from senescing leaves could also lead to enhanced efficiency in foliar Fe fertilization.

Mobile iron species in leaves

Whether to maintain function or prevent harmful effects, the coordination of transition metals in biological systems is always essential. Nevertheless, the coordination of transition metals and the stability of the complexes are influenced by several factors in a highly complex multiequilibrium system such as the biological environment. Thus, ligand concentration and availability, the local pH, the pK_a of the ligand(s), the presence of competing ligands, and the presence of metals other than Fe that also have the potential to form stable complexes together determine the occurrence of any Fe compounds in plants. Fe has a high affinity to, among others, oxygen (O), nitrogen (N), and sulfur (S). With its higher charge-to-size ratio, Fe(III) shows better affinity to negatively charged ligands and especially negatively charged O-ligands, while Fe(II) prefers aromatic N-ligands and S-ligands (Harris, 2002). In aqueous solutions, the deprotonation of the coordinating water molecules of Fe ions is prevalent for Fe(III) even at lower pH values (Hider and Kong, 2013). At neutral pH, Fe(III)-OH complexes crosslink into insoluble ferrihydrite polymers, while Fe(II) will remain soluble even at higher concentrations. Although Fe(II) is susceptible to autoxidation under oxygenic conditions, the reducing environment in the symplast favours maintenance of the reduced status of Fe. Since at neutral pH the concentration of free OH^- ions exceeds the threshold of Fe(III)-OH formation, the maintenance of the reducing environment and thus the provision of available Fe(II) for metabolic processes is essential. In consequence, in a biological system the varying pH conditions require multiple strategies for the coordination of Fe species.

O-ligands of various denticities generally possess lower pK_a values to Fe compared with N-ligands, implying their dominance at lower pH values (Table 1). In apoplastic spaces and acidic compartments of the cells, such as the xylem sap, the apoplast, the vacuole, and the intermembrane spaces of both chloroplasts and mitochondria, the low amount of free OH^- ions does not contest the formation of carboxyl complexes of Fe due to the high abundance of deprotonated carboxyl groups (Martell and Hancock, 1996). Among carboxyl O-ligands, citrate (Cit) and malate (Mal) are prevalent in these spaces. In accordance, Cit and Mal biosynthesis, and their concentrations in the apoplastic aqueous phases, constitutively increase under Fe deficiency (López-Millán *et al.*, 2000; Larbi *et al.*, 2010; Sebastian and Prasad, 2018, Seregin and Kozhevnikova, 2020). Organic acid accumulation in the vacuoles also contributes to the complexing, and thus storage, of Fe under the acidic pH of the vacuoles. (Roschzttardtz *et al.*, 2009; Flis *et al.*, 2016). Cit and Mal may act as tri- and bidentate ligands, respectively, forming stable Fe-chelates, while at mixed ligand composition they also form stable polynuclear complexes (Silva *et al.*, 2009; Flis *et al.*, 2016). At low pH, the Cit complex of Fe(II) is stable, but under moderately acidic conditions it reoxidizes over time and forms stable polynuclear complexes of Fe(III). Thus, taking into account the pH of the apoplast spaces in plants, carboxyl ligands complex Fe(III). Moreover, the formation of stable polynuclear complexes following the reoxidation of Fe(II)-carboxylates would decrease the availability of Fe that is not favoured under biological conditions. Cit has a clear advantage for Fe complexation over Mal, as it requires a 1:2

Table 1. Iron-ligand affinities for relevant complexes in foliar iron homeostasis

Ligand			pK_a*						Complex stoichiometry	Affinity constant (logK) for different stoichiometry†		pH range
Name	Type	Denticity	pK_a1	pK_a2	pK_a3	pK_a4	pK_a5	pK_a6		Fe(II)	Fe(III)	
Cit	O–	3	3.128[a]	4.761[a]	6.396[a]	–	–	–	1:1	4.4[h]	11.5[h]	5.5–6[n]
									1:2	n.d.	32.73[i]	
Mal	O–	2	3.4[b]	5.11[b]	–	–	–	–	1:1	2.6[h]	7.1[h]	n.d.
									1:3	n.d.	n.d.	
Asp	N– O–	3	1.99[b]	3.9[b]	9.9[b]	–	–	–	1:1	5.34[j]	11.4[j]	n.d.
									1:2	8.57[j]	n.d.	
Cys	N– O– S–	2 (3)	1.71[a]	8.36[a]	10.75[a]	–	–	–	1:1	6.69[j]	11.9[j]	~8[d]
									1:2	11.9[j]	14.49[j]	
His	N– O–	3	1.5[a]	6.07[a]	9.34[a]	–	–	–	1:1	5.8[j,‡]	4.4[j,‡]	6–8[o]
									1:2	10.43[j,‡]	n.d.	
Glu	N– O–	3	2.19[c]	4.25[c]	9.67[c]	–	–	–	1:1	3.3[j,‡]	13.7[j,‡]/11.8[k]	5–n.d.[j]
									1:2	n.d.	n.d.	
GSH	S–	1	2.12[b]	3.52[b]	8.67[b]	9.57[b,d]	–	–	1:1	5.12[d]	n.d.	6.5–8[d]
NA	N– O–	6	<1.5[e]	2.35[e]	2.86[f]	6.92[f]	9.14[f]	10.09[f]	1:1	12.8[l]	20.6[f]	6–8[f]
H_2O/OH^-	O–	1	14[g]	–	–	–	–	–	1:1	3.6[m]	11.81[m]	–

* Ionic strength μ=0–0.1 M, at room temperature unless otherwise stated, except glutamate and nicotianamine, where the original publication provided no data.
† Ionic strength μ=0–0.15 M, at room temperature.
‡ Value is corrected according to Martell and Hancock (1996), due to different ionic strength.
References are identified with letters:
[a] Goldberg *et al.*, 2002;
[b] Lide, 2004;
[c] O'Neil, 2013;
[d] Hider and Kong, 2011;
[e] Hider *et al.*, 2004;
[f] von Wirén *et al.*, 1999;
[g] Silverstein and Heller, 2017;
[h] Martell and Smith, 1977;
[i] Silva *et al.*, 2009;
[j] Murphy *et al.*, 2020;
[k] Prasetyo *et al.*, 2020;
[l] Anderegg and Ripperger, 1989;
[m] Martell and Hancock, 1996;
[n] Hanikenne *et al.*, 2021;
[o] Hider *et al.*, 2021.
Asp, aspartate; Cit, citrate; Cys, cysteine; Glu, glutamate; GSH, glutathione; His, histidine; Mal, malate; NA, nicotianamine; n.d., not detectable.

stoichiometry to Fe. In comparison, the Mal concentration has to be at least three times that of Fe to achieve similar complex levels as with Cit (Monsant *et al.*, 2011) (Table 1). Moreover, the concentration of Cit exceeds that of Mal in acidic compartments. Therefore, the formation of Cit complexes of Fe(III) is favoured in plants. Nevertheless, in both the xylem sap and acidic environments of plant cells, amino acids that could compete for Fe are also abundant. As for O-ligands, the amino acids glutamate (Glu) and aspartate (Asp) are also important. Being bidentate Fe ligands, they can effectively compete with other carboxylates for Fe *in vitro* (Table 1) (Aravind and Prasad, 2005; Cui *et al.*, 2020). Nevertheless, as Cui *et al.* (2020) pointed out, based on the glutamate synthase mutant *glu1-4*, Glu complexes of Fe cannot be a major form, especially in the xylem. However, the role of Glu complexes of Fe in organism-level Fe signalling cannot be excluded, but a specific signal requires associated signal perception, too. Nevertheless, the concentration of Cit ensures the dominance of its complexes of Fe, especially in the xylem sap.

At close to neutral pH, the increasing competition of carboxylates with free OH^- ions resulted in enhanced Fe(III)-OH formation and thus Fe precipitation (Martell and Hancock, 1996; Hider and Kong, 2010). In consequence, the coordination of Fe with O-ligands is not favoured in the phloem sap, the cytosol, the matrix of mitochondria, and the stroma of chloroplasts. Indeed, amino acids, particularly histidine (His) and cysteine (Cys), are considered to be effective ligands of Fe under these conditions (Aravind and Prasad, 2005; Seregin

and Kozhevnikova, 2020). Both His and Cys have high affinity to Fe (Table 1) and are able to maintain the reduced state of Fe(II) at neutral pH. Together with other thiol-containing compounds, the S-ligand of Cys has a primary importance to maintain the reduced status of Fe under symplastic conditions (Bhattacharyya et al., 2013; 2019; Murphy *et al.*, 2020). For instance, Cys is a major component in the low-molecular-weight thiol glutathione (GSH). GSH has a high capacity to reduce Fe(III) and serve as a ligand for Fe(II) (Martell and Hancock, 1996; Hider and Kong, 2011). At cytoplasmic pH and concentration, GSH forms complexes with Fe(II) of higher stability than those with Cit (Table 1). Since the cytoplasmic concentration of GSH, in the low mM range, exceeds the concentration of free amino acids (Meyer *et al.*, 2001; Hider and Kong, 2011; Hider *et al.*, 2021), GSH is supposedly the major Fe(II) ligand in the cytoplasm. Moreover, Fe(II)-GSH takes part in Fe–S biosynthesis and in the formation of nitrosyl-Fe complexes. As well as forming peptides such as GSH, amino acids are also important to build proteins. Although amino acid residues are also important ligands of Fe, especially under symplastic conditions (Balk and Pilon, 2011; Balk and Schaedler, 2014), the protein-bound Fe pool is not readily mobilized and thus not significant in the delivery of Fe. One of the few examples might be IRON MAN 1, a long-distance Fe signalling peptide that contains a stretch of Asp residues with the potential affinity to bind and deliver Fe (Grillet *et al.*, 2018).

Along with His, Cys, and GSH, nicotianamine (NA) is an N-ligand compound that is a key component in metal homeostasis of higher plants. NA is derived from methionine through the condensation of three *S*-adenosylmethionine molecules by NA synthase (Curie *et al.*, 2009; Clemens *et al.*, 2013). It has very high affinity to Fe as a hexadentate ligand, forming stable Fe chelates (Table 1). At quasi-neutral pH, Fe-NA complexes are kinetically stable, while at acidic pH total ligand exchange occurs with Cit within 5 min (von Wirén *et al.*, 1999). Removal of cytosolic NA results in impaired intracellular Fe movement in leaves and leads to symptoms of Fe deficiency (Haydon *et al.*, 2012; Lee *et al.*, 2021). Information on the abundance of NA in the symplast is, however, not available. In consequence, it is not clear whether any potential ligands forming Fe complexes with high stability are present in the symplast in a concentration high enough to compete with NA for Fe. Indeed, the lack of information on the intracellular NA concentration suggests that the intracellular NA concentration may be significantly lower than that of GSH. Therefore, it seems that NA supports the intracellular mobility of Fe rather than forming a significant pool of Fe complexes. In NA-deficient *chloronerva* mutants of tomato, Fe phosphate deposits appear (Becker *et al.*, 1995; Liu *et al.*, 1998). Thus, NA is probably involved in keeping liberated Fe soluble (Curie and Briat, 2003). Since no signal of either Fe(II)-NA or Fe(III)-NA can be detected in mature chloroplasts based on Mössbauer spectroscopic analysis (Solti *et al.*, 2012; Müller *et al.*, 2019), the function of Fe-NA complexes seems to be restricted to coping with liberated Fe, but they do not form a major pool. NA and GSH supposedly act as ligands for Fe under different conditions in the symplast, where GSH could be at least in part responsible for multiple aspects of the labile Fe pool, whereas NA might be involved in retaining Fe solubility and in the support of transmembrane Fe transport. Nevertheless, ligand exchange between NA and GSH has not yet been revealed.

Iron transport in leaves

Leaves primarily receive Fe originating from root Fe uptake. In the xylem, Fe is transported predominantly as Fe(III)-carboxylates (Fig. 1) such as $Fe(III)_3$-$(Cit)_3$ (Rellán-Álvarez *et al.*, 2010). Impaired Cit loading into the xylem by Ferric Reductase Defective (FRD) 3 leads to Fe-deficiency symptoms in the shoot (Durrett *et al.*, 2007; Roschzttardtz *et al.*, 2011). Supplying Glu to *frd3* mutant Arabidopsis (*Arabidopsis thaliana*) plants restored leaf chlorosis (Cui *et al.*, 2020), suggesting a role of FRD3 in root-to-shoot Fe transport. Fe translocation towards the youngest leaves is suggested to be based on the sink–source Fe distribution through the phloem. With high enough concentrations in the phloem sap, NA is suggested to be the major ligand in the phloem-based Fe transport towards young leaves and reproductive organs, that is, tissues that are not reached by differentiated xylem vessels (Klatte *et al.*, 2009; Schuler *et al.*, 2012). In Arabidopsis leaves, Yellow Stripe-Like (YSL) 1 and YSL3 (Fig. 1) supposedly transport Fe-NA complexes from veins to surrounding parenchyma cells (Kumar *et al.*, 2017). However, *YSL1* and *YSL3* are expressed only in parenchyma cells and especially in senescing and cauline leaves (Waters *et al.*, 2006), hence we suggest they cannot be responsible for the complete Fe distribution within the whole leaf lamina and may instead function in Fe redistribution. The direction of the Fe transport mediated by YSLs has not been clarified yet. Since plant cells are symplastically connected via plasmodesmata, cell-to-cell Fe movement does not seem to require transporters. Additionally, in acidic compartments, ligand exchange between NA and carboxylates is likely (von Wirén *et al.*, 1999); thus, the functional characterization and contribution of the foliar Fe transport of YSL1 and YSL3 requires further studies. *ysl1ysl3* double mutants fail to induce Fe-deficiency responses in the roots; thus, YSL1 and YSL3 were also proposed to be involved in long-distance Fe status signalling (Kumar *et al.*, 2017). The xylem sap infiltrates the apoplast, where Fe(III)-carboxylates are still the dominant Fe species (Fig. 1). Leaf cells operate a reduction-based Fe uptake utilizing both Fe(III)-Cit and Fe(III)-Mal (Fig. 1 and 2; Brüggemann *et al.*, 1993; Larbi *et al.*, 2001). In Arabidopsis, FRO6 targets the plasma membrane of mesophyll cells (Jeong *et al.*, 2008). Overexpression of *AtFRO6* in tobacco (*Nicotiana tabacum*) leads to increased tolerance of leaf Fe chlorosis (Li *et al.*, 2011). Ascorbate (Asc)-mediated reduction of Fe(III) was suggested as an obligatory step in Arabidopsis embryos

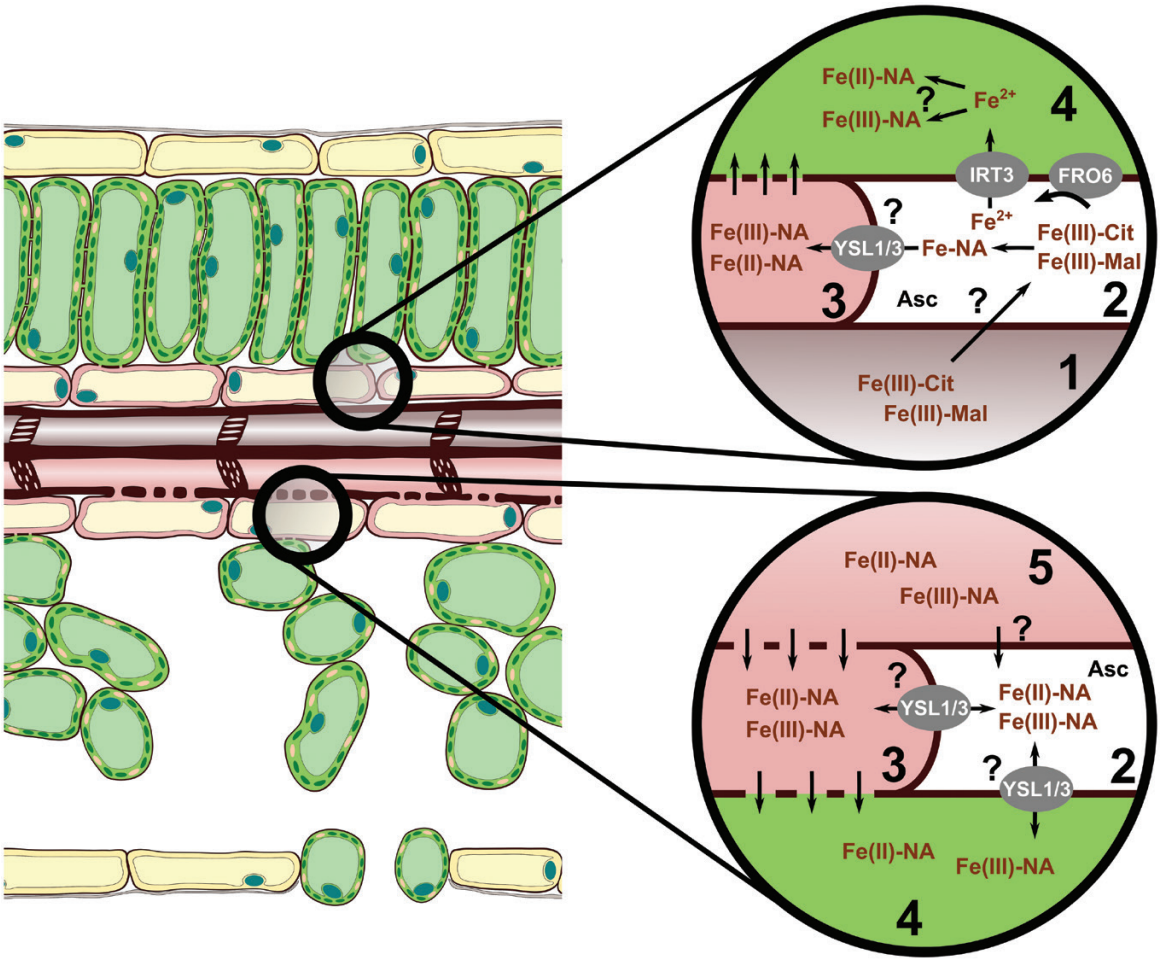

Fig. 1. Fe transport processes in leaves. Leaves of dicot plants such as Arabidopsis primarily receive Fe through the vasculature via root-to-shoot Fe translocation in the xylem vessels or from source–sink Fe redistribution processes operated by the phloem. Fe(III)-Cit is the dominant species of Fe in the xylem (1) that reaches the leaf tissues. Fe-carboxylates originating from the xylem sap are thought to infiltrate the intracellular spaces and the apoplast (2). Although under the slightly acidic environment in the apoplastic spaces Fe(III)-carboxylates have a high stability, ligand exchange towards NA has not been clarified yet. Parenchyma cells around the vasculature (3) express YSL1 and YSL3, thus Fe-NA uptake is also suggested. Mesophyll cells (4) primarily operate a reduction-based method of Fe acquisition. FRO family enzymes can utilize Fe(III)-carboxylate complexes; Asc-mediated reduction and photoreduction of Fe(III)-carboxylates might be also involved, as shown by the redundant nature of the ferric chelate reductase activity of the mesophyll. In addition to Fe translocation in the xylem, the phloem (5) is also involved in the redistribution and transport of Fe, although the magnitude of xylem transportation is supposedly significantly higher. In all cytoplasm-filled environments, including the phloem, the presence of Fe-NA species is suggested to keep Fe soluble. Moreover, the transport of Fe species involved in long-distance Fe signalling may also use the phloem transportation pathway. The nature of phloem unloading with respect to Fe species has not been clarified yet. Cells that are symplastically connected through plasmodesmata might not need special Fe transporters. Indeed, the presence of YSL transporters, especially during leaf development and at senescence initiation, indicate that the transport of Fe-NA species is also important in the phloem transport of Fe. For abbreviations and further details see the text.

prior to Fe uptake (Grillet *et al.*, 2014). Since Asc is present in the apoplast of vascular parenchyma facing xylem vessels in the leaves (Zechmann *et al.*, 2011), xylem unloading and Fe acquisition by the vascular parenchyma cells may rely in part on Asc-mediated reduction (Figs 1, 2). Nevertheless, in leaves as in aerial organs, the photoreduction of Fe could be an important factor that contributes to Fe acquisition. The contribution of light-irradiance-induced reduction to the Fe uptake into mesophyll cells has not been characterized yet. Once Fe is reduced, Iron Regulated Transporter (IRT) 1 and IRT2 have a primary role in transporting Fe(II) into the cytoplasm (Figs 1, 2). His residues on the cytosolic side of IRT1 are suggested to bind Fe, affecting its function (Cointry and Vert, 2019). In contrast to the situation in the roots, the expression of IRT1 is not enhanced by Fe deficiency in the leaves, which indicates a rather stable uptake by leaf cells (Bauer *et al.*, 2004). Although there is clear evidence that Fe-NA transporters are also involved in the distribution and redistribution of Fe, the functional characterization of this transport is still elusive.

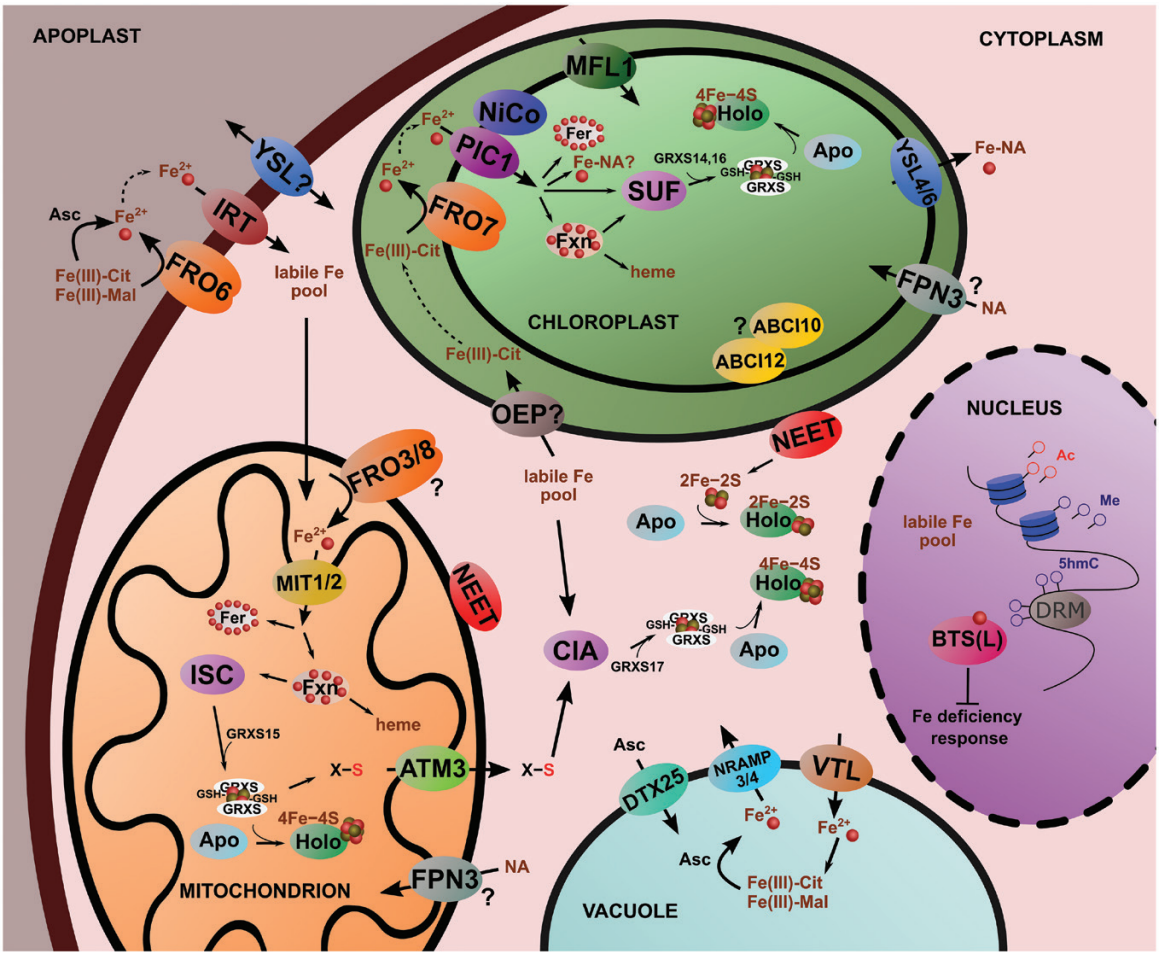

Fig. 2. Fe homeostasis of mesophyll cells. Mesophyll cells of dicots such as Arabidopsis primarily operate a reduction-based Fe acquisition mechanism, whereas the extent of YSL-mediated Fe uptake is less well characterized. IRT-type transporters are suggested to mediate the transport of divalent metals. Although transmembrane transport is generally accepted to be based on the free forms, Fe should be complexed in the cytoplasm to avoid ROS generation. Fe complexed by low-molecular-weight ligands is thought to be part of the labile Fe pool of the cells. The proper composition of this labile Fe pool is yet to be understood. FRO family enzymes are targeted into both chloroplasts and mitochondria, thus their Fe acquisition is supposedly dominated by the reduction-based pathway, too. Fe import into the mitochondrial matrix and chloroplast stroma is a complex process because of the double-envelope system of the organelles. Nevertheless, functional characterization of the protein members of this machinery is far from complete, as discussed in Vigani *et al.* (2019). In Fe acquisition by chloroplasts, PIC1, NiCo, and MFL components can cooperate, whereas mitochondria may primarily operate MIT1 and MIT2. Fe in the chloroplasts and mitochondria is directed towards incorporation into hemes and Fe–S clusters. Hemes are synthesized by ferrochelatases, but frataxin is considered to be involved in heme biosynthesis in mitochondria. Mitochondria and chloroplasts operate the ISC and SUF systems, respectively, for the biogenesis of Fe–S clusters. Plant GRXs are involved in the management and insertion of Fe–S clusters into apoproteins. The export of Fe–S clusters towards the cytoplasm involves NEET proteins and ABC transporters. Both the photosynthetic and respiratory electron transport chains require a significant amount of Fe, thus in mesophyll cells, Fe is primarily directed towards protein complexes operating these processes. During the decomposition of these systems, Fe can be liberated. YSL family transporters are considered to be involved in retaining the solubility of Fe and exporting Fe out of the plastids. In addition to the organellar Fe–S cluster biosynthesis, in the cytoplasm the eukaryotic CIA system, which partly depends on the mitochondrial ISC system as a source of reduced sulfur, is operational. Although the vacuoles of the leaf cells contribute to the temporal storage of Fe, the primary Fe storage that helps to manage temporal Fe excess or Fe liberation are ferritins (also illustrated in Fig. 3). Since Fe is a potentially toxic element, proper control over cellular Fe status is essential. Hemerythrin domain proteins BTS(L) were described as Fe sensors of plant cells. Nevertheless, the complexity of Fe-status-connected responses in plant cells suggests the existence of multiple sensing and regulatory mechanisms, mediated by small molecules and Fe–S sensing mechanisms. Although the understanding of epigenetic mechanisms that regulate cellular Fe homeostasis is far from complete, both DNA methylation by Domains Rearranged Methyltransferases (DRM) and histone modifications are important signals. For abbreviations and further details see the text.

Iron in cofactors in leaves

Mesophyll cells take up a significant amount of Fe, where it is directed towards Fe-containing cofactors of redox enzymes. Thus, the largest proportion of Fe in leaves is incorporated into tetrapyrrole and Fe–S cofactors. Tetrapyrroles are crucial in all cell compartments for various electron transfer reactions such as respiratory and photosynthetic electron transport chains

(Brzezowski *et al.*, 2015). Sirohemes, synthesized on the first branch of the tetrapyrrole pathway, are harboured by plastidial nitrite and sulfite reductases (Tripathy *et al.*, 2010; Askenasy and Stroupe, 2020). Hemes are synthesized from protoporphyrin IX with the insertion of Fe(II) by ferrochelatase (Solymosi and Myśliwa-Kurdziel, 2021). The direct mechanism of Fe donation during heme biosynthesis is unknown, although frataxin has been suggested to play a role in it (Fig. 2) (Maliandi *et al.*, 2011; Gomez-Casati *et al.*, 2018; Armas *et al.*, 2019). Free hemes are stable, thus they can contribute to the formation of the Fe pool in cells (Shviro and Shaklai, 1987; Sahini *et al.*, 1996; Hanna *et al.*, 2016). In the cytosol, hemes form 1:1 complexes with GSH (Hider and Kong, 2011; O'Keeffe *et al.*, 2021). Moreover, plastidial, cytosolic, and nuclear proteins have been also identified as heme-binding proteins (Shimizu *et al.*, 2020; Sylvestre-Gonon *et al.*, 2020). Since the ^{57}Fe Mössbauer spectroscopy characteristics of hemes and 4Fe–4S clusters are hardly distinguishable, direct determination of the proportion of Fe incorporated into hemes is limited.

Fe–S clusters represent more electronegative cofactors when compared with hemes. Rhombic 2Fe–2S and cubane 4Fe–4S clusters are common in various proteins (for review, see Przybyla-Toscano et al., 2018, 2021). In chloroplasts and mitochondria, the photosynthetic and respiratory electron transport chains, respectively, have the highest demand for Fe–S clusters. The majority of Fe in the chloroplasts can be identified as 4Fe–4S clusters (Solti *et al.* 2012). Fe–S clusters are sensitive to molecular oxygen and unstable in aqueous solutions, and thus their coordination through Cys or His residues is essential. In all eukaryotic cells, both the mitochondrial iron–sulfur cluster assembly (ISC) pathway and the eukaryotic cytosolic iron-sulfur assembly (CIA) pathway are operational, whereas plants also operate the sulfur mobilization (SUF) system in their plastids (Fig. 2). Indeed, the CIA pathway is heavily dependent on mitochondria (Balk and Pilon, 2011; Lu, 2018). Frataxin serves as an Fe donor for the mitochondrial ISC system (Fig. 2). In plants, lack of frataxin is lethal, while knockdown mutants show impaired activity of Fe–S-containing mitochondrial enzymes (Gomez-Casati *et al.*, 2018; Armas *et al.*, 2020). Turowski *et al.* (2015) first reported that plant frataxin is double-localized to the chloroplasts too, hypothetically donating ferrous Fe for the plastidial SUF system (Fig. 2). The biosynthesis of 4Fe–4S clusters in mitochondria depends on a reduction step in which Ferredoxin (FDX) 2 provides the reducing power. In this reductive fusion of 2Fe–2S clusters, FDX1 cannot replace FDX2 (Weiler *et al.*, 2020). GSH can bind 2Fe–2S clusters in cytoplasmic conditions. GSH depletion impairs cytosolic Fe–S protein maturation and increases the Fe levels in the mitochondria (Kumar *et al.*, 2011; Qi *et al.*, 2012). Defects in GSH biosynthesis lead to the down-regulation of the essential Fe homeostasis genes *Natural Resistance-Associated Macrophage Protein* (*NRAMP*) *3 and NRAMP4, Permease In Chloroplast* (*PIC*) *1*, *Ferritin* (*FER*) *1*, and *IRT1* in Arabidopsis (Shee *et al.*, 2021, Preprint).

Monothiol (Class II) GRXs with Fe–S cluster transferase activity are also important in delivering Fe–S clusters (Berndt and Lillig, 2017; Trnka *et al.*, 2020; Talib and Outten, 2021). In Arabidopsis, Class II GRXS17, GRXS15, and GRXS14/16 are located in the cytoplasm, mitochondria, and chloroplasts, respectively (Fig. 2). Plant GRXS15 can restore mitochondrial Fe homeostasis in yeast *grx5* mutants (Couturier *et al.*, 2015). However, information on yeast GRXs cannot be directly translated to plant models. Although yeast Class II GRXs have a central role in the sensing of Fe–S clusters in mitochondria (Mühlenhoff *et al.*, 2020), such a role of plant GRXs has not been confirmed so far. Knockout mutation of Arabidopsis mitochondrial GRXS15 is lethal due to defective embryo development, whereas the mutation of plastidial GRXs affects the sensitivity to oxidative stress (Balk and Pilon, 2011; Moseler *et al.*, 2015). The fact that mutation of plastidial GRXs is not lethal suggests that the integration of Fe–S clusters into the apoproteins of the photosynthetic apparatus might be an independent action. Plant GRXs can form heterodimers with BOLA proteins (GRXS14/BOLA1 in plastids and GRXS15/BOLA4 in mitochondria; Roret *et al.*, 2014; Rey *et al.*, 2019). Although in yeast GRX/BOLA heterodimers are involved in Fe sensing by affecting gene expression (Rey *et al.*, 2019), such a role has not been documented in plants so far. In yeast, Fe–S clusters are proposed to be exported from mitochondria in the form of [2Fe–2S] $(GS)_4^{2-}$ by ATM1 (Li and Cowan, 2015). However, Fe–S clusters are not stable in aqueous solution under aerobic conditions. Thus, ATM1 most likely transports an S-containing compound (Lill and Freibert, 2020). Regarding plant ATM3, Schaedler *et al.* (2014) proposed and Marty *et al.* (2019) confirmed that plant mitochondrial ATM3 exports GSH and persulfide-containing glutathione polysulfide, thus affecting cytoplasmic Fe–S cluster biosynthesis (Fig. 2). Moreover, ATM3 is indirectly involved in cyclic pyranopterin monophosphate (Moco cofactor intermediate) biosynthesis (Kruse *et al.*, 2018). Thus, ATM3 represents a hub linking Fe and N metabolism. Nevertheless, neither *atm3* nor *grxs17* is lethal in Arabidopsis (Balk and Pilon, 2011; Iñigo *et al.*, 2016); thus, the operation of the CIA system for the cytoplasmic biosynthesis of Fe–S clusters is not an exclusive source of the cofactors in the cytoplasm, and GRXS17 primarily operates in the maturation of cytoplasmic Fe–S proteins (Martins *et al.*, 2020). Recent results (Cheng *et al.*, 2020) on the expression of the Fe-responsive pathway elements showed, however, that GRXS17 can be also involved in the signalling of both the redox and Fe status. Since Fe liberation has a pivotal role in generating oxidative stress, the management of Fe–S clusters by GRXs is crucial in protecting them from Fe liberation. In consequence, redox signals can also report on improper Fe–S cluster metabolism.

Although the connections between the cytoplasmic and organellar Fe–S cluster metabolisms have not been revealed completely in plants, the comparison of results obtained in

Δatm3, *Δgrxs17*, and *NEET-H89C* lines indicate that chloroplasts are important sources of Fe–S clusters for cytoplasmic proteins. Since NEET proteins are involved in the donation of 2Fe–2S clusters to cytoplasmic acceptors, they are suggested to have a role in the transfer of Fe–S clusters to cytoplasmic apoproteins (Karmi *et al.*, 2018; Zandalinas *et al.*, 2020; Nechushtai *et al.*, 2020). In Arabidopsis, NEET is double-localized to mitochondria and chloroplasts (Fig. 2; Nechushtai *et al.*, 2012; Su *et al.*, 2013; Khan *et al.*, 2018). Loss of NEET function is detrimental and results in enhanced Fe overaccumulation and reactive oxygen species (ROS) production, developmental retardation, enhanced senescence, increased sensitivity to low Fe nutrition, and decreased sensitivity to high Fe nutrition (Nechushtai *et al.*, 2012; Lu, 2018; Zandalinas *et al.*, 2020). The mechanism involved in the sensing of Fe–S clusters is also under debate in photosynthetically active plant cells.

Interaction between Fe(II) or Fe(III), nitric oxide (NO), and small-molecular-weight thiols such as GSH yields mononitrosyl Fe complexes (MNICs) and dinitrosyl Fe complexes (DNICs) (Lewandowska *et al.*, 2011; Li and Li, 2016). DNICs are formed during the attack by NO on Fe–S clusters that induces the release of Fe from the clusters (Landry *et al.*, 2011; Berndt and Lillig, 2017; Tewari *et al.*, 2021). GSH complexing of DNICs results in the increased stability of these species (Vanin, 2009). The connection between plant cells through plasmodesmata enables the symplastic transport of DNICs, so they could be also involved in medium/long-distance Fe delivery and signalling. Nevertheless, the effects of NO on Fe homeostasis seems to be tissue and developmental stage specific. In these signalling events, the connections of the nitrosative and Fe-sensing-induced signalling remain unresolved at multiple points.

Compartmentalization of iron in leaves

The labile Fe pool is the source of Fe for organellar Fe uptake and cytoplasmic biosynthesis of cofactors (Fig. 2). Roschzttardtz *et al.* (2013) reported that, under normal Fe supply, Fe in the leaves is primarily localized to the chloroplasts of the spongy mesophyll cells and in the vasculature. Under supraoptimal Fe supply, the accumulation of Fe in these locations is more pronounced. Immune localization revealed that under normal Fe supply, ferritin is mainly located in the xylem-associated cells but not in mesophyll cells (Roschzttardtz *et al.*, 2013); thus, the presence of Fe in the chloroplasts of the spongy parenchyma cells corresponds to Fe that has already been incorporated into the photosynthetic machinery. The high-efficacy Fe uptake of chloroplasts relies on the reduction-based mechanism (Solti *et al.*, 2014; Ságí-Kazár *et al.*, 2021). This machinery is thought to include the chloroplast inner envelope membrane proteins PIC1/Translocon of Inner Chloroplast envelope 21, Nickel-Cobalt Transporter, and Ferric Reductase Oxidase (FRO) 7 (Duy et al., 2007, 2011; Jeong *et al.*, 2008; Fig. 2). Although Asc is relatively abundant in chloroplasts and can reduce Fe(III) directly, it does not facilitate the reduction-based acquisition of Fe (Solti *et al.*, 2012). Since Fe(II) was not detected by ^{57}Fe Mössbauer spectroscopy either in the intermembrane space or in the chloroplast stroma during Fe uptake (Solti *et al.*, 2012), the direct loading of Fe into transporters following the reduction step suggests the close collaboration of the Fe(III) reduction and Fe uptake machinery. This model also explains why externally applied Asc does not facilitate Fe uptake. Ferroportin (FPN) 3 (synonymous to IREG3 and MAR1; Conte *et al.* 2009) was identified as a double-localized transporter in both mitochondria and plastids (Fig. 2) (Kim *et al.*, 2021). FPN3 was suggested to be involved in NA uptake into the plastids (Conte and Lloyd, 2010; Duy *et al.*, 2011). Although in the shoot FPN3 expression is independent of the Fe status, it was suggested to have a role in Fe mobilization from organelles (Kim *et al.*, 2021). This role of NA in the chloroplasts is also supported by the Fe precipitation in the chloroplast stroma in NA-defective *chloronerva* tomatoes, as discussed above. Divol *et al.* (2013) showed that the Fe-NA transporters YSL4/YSL6 are involved in the Fe transport of plastids in germinating seeds, while Fe accumulates in the chloroplasts of *ysl4ysl6* double mutants. Nevertheless, *YSL4* expression remained low in *Brassica napus* leaves (Müller *et al.*, 2019) and no data are available on the functional characterization of YSL4/YSL6; it has been suggested they may have a role in Fe release from plastids (Fig. 2). Similarly, Mitoferrin-Like 1 (MFL1) (Fig. 2) remains a less well characterized component of the Fe homeostasis of chloroplasts (for review, see Vigani *et al.*, 2019). The plastidial, soluble, ATP-binding ABC-transporter subunits ABCI10 and ABCI11/NAP14 are also strongly associated with the Fe homeostasis of chloroplasts (Fig. 2) (Voith von Voithenberg *et al.*, 2019). The inner-envelope-associated ABCI10 also interacts with the membrane-intrinsic ABCI12, and this complex was suggested to be a part of an energy-coupled transporter unit (Voith von Voithenberg *et al.*, 2019). Nevertheless, the characterization of the mechanism of action of this system, including the direction of the transport, is still incomplete. Although the release of Fe from chloroplasts is evident during senescence (Barton, 1970; Pottier *et al.*, 2019; Pham *et al.*, 2020; Ságí-Kazár *et al.*, 2021), it is not clear whether the export of Fe or Fe cofactors occurs. Moreover, the transport system that would be responsible for Fe release is also under debate. Nechushtai *et al.* (2020) suggests that the outer-envelope-anchored plastidial NEET may be involved in the delivery of 2Fe–2S clusters to cytoplasmic proteins. Nevertheless, the 2Fe–2S cluster transfer activity is restricted to cytoplasmic proteins, whereas no transmembrane transport activity has been confirmed so far, and thus its contribution to the export of Fe–S clusters out of the chloroplasts remains questionable.

In aerial tissues, 80–90% of Fe is found in chloroplasts (Terry and Abadía, 1986), especially in photosystem (PS) I, PSII, and cytochrome b_6f complexes of the photosynthetic electron transport chain, but also in redox enzymes of the nitrite and sulfate

assimilation pathways (for review, see Hantzis *et al.*, 2018). Non-heme mononuclear Fe is an essential cofactor of the photosystem PSII core complex bound on His residues (Kato *et al.*, 2021). Therefore, Fe accumulation in chloroplasts has a primary importance in the Fe nutrition of mesophyll cells. According to chloroplast Fe uptake measurements, no Fe environments can be identified other than 4Fe–4S clusters, indicating that after Fe is taken up into the chloroplasts it is directly incorporated into Fe–S clusters (Solti *et al.*, 2012). Zhang *et al.* (2021) demonstrated that DJA5 and DJA6, mutation of which is lethal, bind Fe at conserved Cys residues and transfer it towards the SUF machinery. Under Fe deficiency, the SUF apparatus becomes suppressed due to down-regulation of *SUFA* and *SUFB* (Hantzis *et al.*, 2018; Sági-Kazár *et al.*, 2021), indicating that the SUF machinery stays under the regulation of intracellular Fe status. Moreover, chlorophyll accumulation is also tightly linked to Fe–S cluster biosynthesis (Hu *et al.*, 2017). Among the components of the photosynthetic machinery, cytochrome b_6f and PSI are also highly affected by Fe deficiency (Hantzis *et al.*, 2018). Indeed, the amount of PSI supercomplexes depends strongly on Fe status (Andaluz *et al.*, 2006; Timperio *et al.*, 2007; Basa *et al.*, 2014). When the incorporation of Fe into the photosynthetic apparatus is restricted, such as under etiolation or during senescence, or when there is an excess of Fe, ferritins become the most significant Fe-containing components in chloroplasts (Fig. 3) (Roschzttardtz *et al.*, 2013; Hantzis *et al.*, 2018; Chen *et al.*, 2019). In Arabidopsis, FER1 and FER3 are plastidial proteins, FER4 is localized to plastids and mitochondria in vegetative

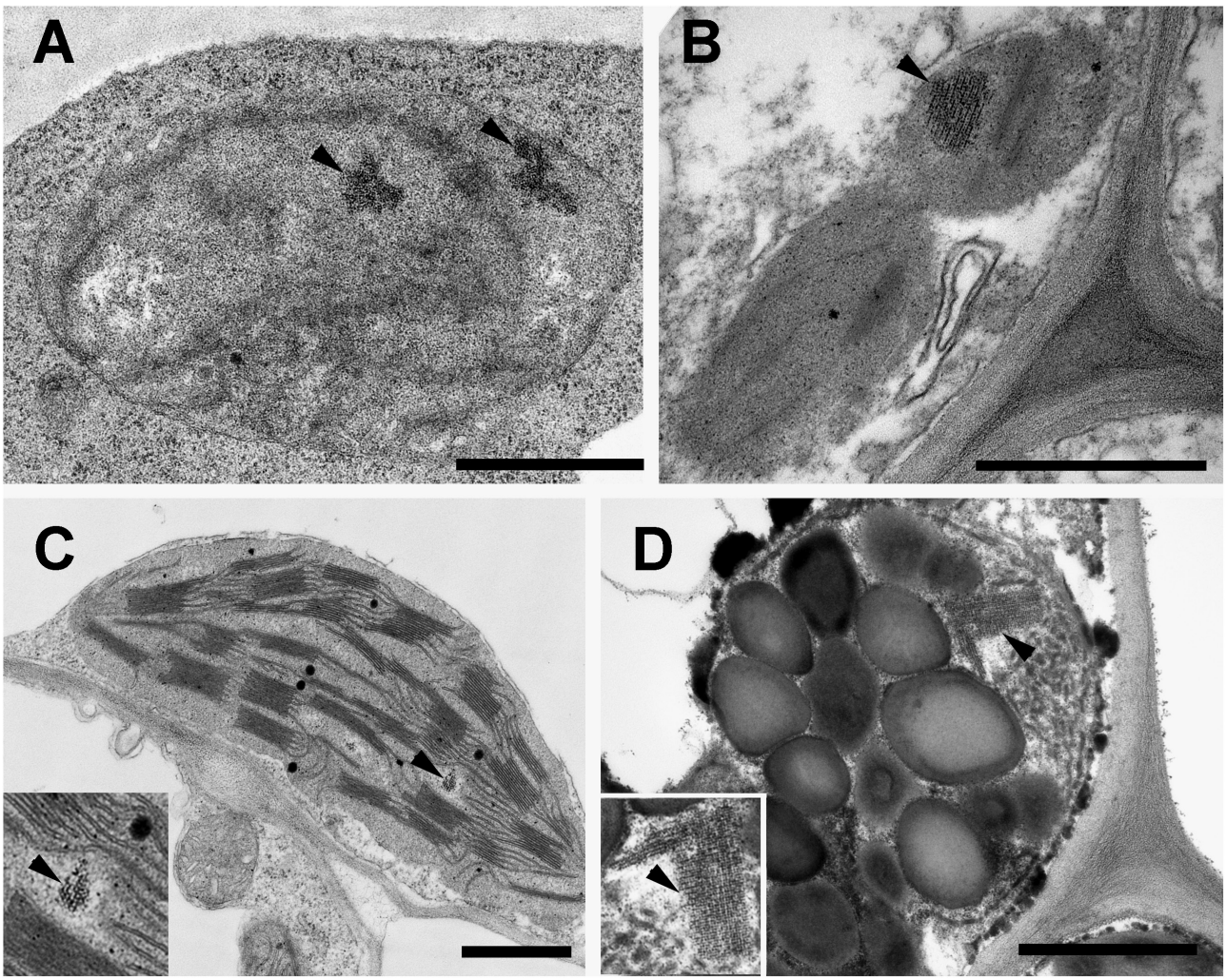

Fig. 3. Localization of ferritin om various plastid types, cells and tissues of dicots. (A) Ferritins in an etio-chloroplast of a mesophyll cell in the outer leaf primordium of the fully closed bud of common ash (*Fraxinus excelsior*) (for further details see Solymosi *et al.*, 2012). (B) Ferritin from a plastid located in the phloem parenchyma of the leaves of 2-week-old dark-forced rosemary (*Rosmarinus officinalis*) shoot (for details see Böszörményi *et al.*, 2020). (C) Chloroplast with ferritin from the leaves of 2-week-old photosynthetically active light-grown pea (*Pisum sativum*). (D) Senescing chloroplast from the mesophyll (spongy parenchyma) cells of senescent *Parthenocissus tricuspidata* leaf. The arrowheads point to ferritin, and the insets show magnified views of the ferritin region of the plastids. Electron microscopic sample preparation and analysis were performed as described in Böszörményi *et al.* (2020). Scale bars=1 μm.

tissues, whereas FER2 is found exclusively in seed plastids (Fig. 2) (Petit *et al.*, 2001 Theil and Briat, 2004; Ravet *et al.*, 2009; Zhao, 2010; Theil, 2013). Since no ferritin-related Fe species were detected by ^{57}Fe Mössbauer spectroscopy in chloroplasts originating from developed, non-senescent leaves of plants with optimal Fe nutrition (Solti *et al.*, 2012), the induction of ferritin-based Fe storage is negligible under these conditions. Instead, ferritins are involved in protection against oxidative damage by sequestering Fe in an inert form to avoid Fenton reactions (Briat and Lobréaux, 1997; Briat *et al.*, 1999; Ravet *et al.*, 2009). This protective function is important in the case of local or temporal excess of Fe (Izaguirre-Mayoral and Sinclair, 2009; Briat *et al.*, 2010; Roschzttardtz *et al.*, 2013). Ferritins have been observed by transmission electron microscopy in various cell types with photosynthetic apparatus that is not fully active, such as plastids in xylem- or phloem-associated parenchyma (Fig. 3) (Tarantino *et al.*, 2003; Böszörményi *et al.*, 2020; Roschzttardtz *et al.*, 2013). Ferritin also occurs in plastids of etiolated organs such as leaf primordia of cabbage (*Brassica oleracea*) (Solymosi *et al.*, 2004) or buds (Fig. 3) (Solymosi *et al.*, 2012). Temporal Fe excess in plastids, and thus ferritin accumulation, is observed during normal plant development, including leaf senescence (Fig. 3) (Tarantino *et al.*, 2003; Solymosi *et al.*, 2004), but also under various stress conditions (Seckback, 1982). During senescence, ferritins operate as temporary storage of Fe released from the breakdown of the photosynthetic apparatus before its remobilization (Fig. 3). Frataxin seems to have an overlapping role with ferritins in keeping cellular Fe levels under control (Ramirez *et al.*, 2011; Murgia and Vigani, 2015). Nevertheless, the relationship of ferritin- and frataxin-based Fe storage in cells is not clear in the leaf. Ferritin-based Fe storage in plastids, indeed, seems to be associated with situations involving a high risk of Fe liberation. Thus, ferritins are predominant in binding Fe in the organelles, especially in chloroplasts, whereas mobile Fe ligands such as NA are instead involved in the mediation of Fe loading to and release from ferritins.

In mitochondria, elements of the respiratory electron transport chain also require a significant amount of Fe in the form of cofactors (for review, see Vigani and Hanikenne, 2018). Similarly to plastids, the Fe transport activities of mitochondria also rely on the reduction-based strategy (Fig. 2). *In silico* analysis predicts two FRO proteins (FRO3 and FRO8) that target mitochondria in Arabidopsis (Mukherjee *et al.*, 2006; Connolly *et al.*, 2018). FRO3, which is also expressed in the vascular cylinder of roots (Mukherjee *et al.*, 2006), is abundant during leaf development, but the accumulation of FRO8 is restricted to senescence in shoots; thus, a functional split between mitochondrial FROs exists (Vigani *et al.*, 2019). In contrast to chloroplast FRO7, which is localized in the inner envelope of chloroplasts (Solti *et al.*, 2014), *in silico* analysis suggests that FRO3 may target the outer membrane of mitochondria and thus can utilize cytosolic NADH (Connolly *et al.*, 2018). Indeed, these preliminary data require validation. Altogether, functional analysis of mitochondrial FRO enzymes remains incomplete. Once Fe(III) is reduced, it should cross the mitochondrial membranes. A mitochondrial Fe transporter (MIT) has been characterized in rice (*Oryza sativa*) (Bashir *et al.*, 2011; Vigani *et al.*, 2016). Arabidopsis expresses two Mitoferrin (MIT) proteins, MIT1 and MIT2 (Fig. 2; Jain *et al.*, 2019). The *mit1* and *mit2* single mutants show no visible phenotype, whereas the *mit1mit2* double mutation is lethal (Jain *et al.*, 2019). It is important to note that a significant proportion of available data on mitochondrial Fe homeostasis has been obtained from root cells, and validation of these data is also required in photosynthetically active cells.

Mesophyll cells are characterized by the presence of a central vacuole, which is involved in the regulation of cellular Fe homeostasis (Gayomba *et al.*, 2015; Vigani *et al.*, 2019; Bashir *et al.*, 2021). Fe loading into the vacuole is primarily managed by Vacuolar Iron Transporter (VIT) proteins (Eroglu *et al.*, 2019). In rice, *OsVIT1* is expressed in developing leaves (Zhang *et al.*, 2012; Che *et al.*, 2021). Functionally redundant VIT-like (VTL) transporter genes *VTL1*, *VTL2*, and *VTL5* are expressed in vegetative tissues (Fig. 2) (Gollhofer *et al.*, 2014). Knowledge of the exact histological location of VIT and VTL proteins in leaves is still scarce, although VIT1 was shown to be active in the xylem parenchyma cells of Arabidopsis embryos (Kim *et al.*, 2006). Although clear evidence is missing on the vacuolar Fe compounds, it is likely that Fe(III) associates with carboxylates (Roschzttardtz *et al.*, 2009; Flis *et al.*, 2016; Vigani *et al.*, 2019). NRAMP3 and NRAMP4 are involved in the retrieval of Fe (Fig. 2) (Lanquar et al., 2005; 2010; Bastow *et al.*, 2018). Although vacuolar Fe exporters are suggested to be divalent metal carriers, the mechanism of Fe reduction for the transport is still unclear. So far, no FRO enzymes have been identified in the tonoplast membrane in Arabidopsis, although OsFRO1 was shown to act as a vacuolar ferric chelate reductase in rice leaves (Li *et al.*, 2019). Overexpression of OsFRO1 leads to increased Fe sensitivity, while Fe excess leads to the down-regulation of OsFRO1 (Li *et al.*, 2019). Asc-mediated Fe reduction can be also important in mobilizing vacuolar Fe. In Arabidopsis, the MATE family member DTX25 was shown to transport Asc across the tonoplast membrane, and its mutation caused sensitivity to Fe deficiency during germination (Fig. 2) (Hoang *et al.*, 2021). As a consequence, higher plants seem to transport reduced Fe across the tonoplast membrane. The means of Fe accumulation and reduction is believed to be taxon dependent, but the transport of reduced Fe is supported by evidence. Vacuolar Fe storage becomes important when ferritin-based storage in the organelles is limited, but it can be also involved in Fe reallocation processes associated with the degradation of cell compartments, discussed below.

Information on the intracellular iron status in leaves

Fe uptake and allocation in the cells require the sensing of intracellular Fe status, inducing a reciprocal interaction between sensing and regulation. In contrast to root cells, which take up Fe and translocate it to the places where it is utilized,

leaf cells—especially during leaf development—are the sink of Fe trafficking. Since the overaccumulation of Fe, especially in the presence of ROS, can lead to toxicity, controlling this accumulation is essential. Taking into account the complexity of cellular Fe homeostasis, and physiological and developmental responses to Fe, multiple Fe-sensing mechanisms should be involved in the fine-tuning of Fe homeostasis (Miller and Busch, 2021). Conserved mechanisms in protein-based Fe signalling in eukaryotic cells suggest that the mechanism of monitoring intracellular Fe status has a common nature.

Although the nature and composition of the labile Fe pool in the cytoplasm is still under debate, sensing the available Fe pool is of primary importance. In the sensing of intracellular Fe, O-bridged di-Fe clusters bound to hemerythrin-type proteins have a primary importance. Hemerythrin motif-containing RING and zinc-finger proteins (HRZs) in rice and BRUTUS (BTS) E3 ubiquitin ligases in Arabidopsis are negative regulators of Fe deficiency responses (Fig. 2) (Long *et al.*, 2010; Hindt *et al.*, 2017; Kobayashi, 2019; Riaz and Guerinot, 2021). The function of Arabidopsis BTS is redundant to that of its paralogs, BTS-Like (BTSL) 1 and BTSL2, but in leaves, only *BTS* is expressed (Rodríguez-Celma *et al.*, 2019). Based on the tissue-specific differences in the expression of hemerythrin-type sensors, slight alterations in the Fe signalling among root and shoot tissues can be expected. Fe binding to BTS directs IVc basic helix-loop-helix (bHLH) transcription factors, upstream regulators of Fe signalling, to degradation (Rodríguez-Celma *et al.*, 2019; Gao and Dubos, 2021). The expression of *HRZs/BTS* is strongly induced by Fe deficiency in shoot tissues (Rodríguez-Celma *et al.*, 2013; Kobayashi *et al.*, 2013; Hindt *et al.*, 2017). Although the Fe signalling cascade of bHLH transcription factors is well established in roots, the role of bHLH transcription factors in the regulation of Fe homeostasis in leaves remains controversial. The *in silico* analysis of Hantzis *et al.* (2018) indicated that major Fe homeostasis effector elements in leaves such as *SUFB* and *Ferredoxin 2* have no or a very limited probability of bHLH binding to their promoter regions. It is also an open question whether organellar Fe status regulates the expression of nuclear genes directly.

In the Fe homeostasis of leaf cells, signals derived from the Fe and the associated metabolic status of organelles could have primary importance. Both chloroplasts and mitochondria synthesize 3′-phosphoadenisine-5′-phosphate (PAP), which is involved in retrograde signalling (Estavillo *et al.*, 2011). Mutation of the PAP system induces constant activation of the root Fe acquisition system together with higher shoot Fe accumulation (Balparda *et al.*, 2020). Since organelles are the primary sites of Fe incorporation and cofactor biosynthesis, the saturation of which is required for a balanced metabolism, the PAP system is a supposed member of the retrograde signalling system that also delivers information on the Fe status. Nevertheless, operation of the PAP system needs further investigations in photosynthetically active cells. The elimination of formate also seems to be linked to the signalling of organellar Fe status. Formate is produced in the methionine cycle and is involved in NA biosynthesis. Its detoxification relies on Formate Dehydrogenase (FDH), a double-localized protein in the mitochondria and chloroplasts. Together with NA biosynthesis, FDH is also induced by Fe deficiency (Suzuki *et al.*, 1998); the FDH promoter is sensitive to Fe status, and changes in *FDH* expression lead to altered Fe accumulation in aerial tissues (Vigani *et al.*, 2017, Di Silvestre *et al.*, 2021). In consequence, formate can act as an indirect signal of intracellular Fe status.

In addition to PAP and formate, NO metabolism is also linked to Fe status signalling in leaves. Upon Fe excess, rapid NO accumulation occurs in the chloroplasts (Arnaud *et al.*, 2006). Excess Fe was shown to decrease the expression of *FRO7* (Sági-Kazár *et al.*, 2021). Forming *S*-nitrosoglutathione adduct with GSH, a nitrosative signal triggers the Fe acquisition system in roots (Kailasam *et al.*, 2018). Under optimal Fe nutrition, overexpression of *S*-nitrosoglutathione reductase (GSNOR), which eliminates *S*-nitrosoglutathione and thus the nitrosative signal, was associated with a decrease in the expression of *FER1*, *FER2*, *PIC*, *FRO7*, and *VIT* in mature leaves of tomato (Wen *et al.*, 2019). In contrast, under Fe deficiency, overexpression of *GSNOR* significantly up-regulated the expression of *FRO7* (Wen *et al.*, 2019). Since in developed leaves the expression of *FRO7* is suppressed under conditions of both Fe deficiency and Fe excess (Sági-Kazár *et al.*, 2021), the nitrosative signal seems to be a negative regulator of the expression of plastidial Fe acquisition elements. Although NO biosynthesis in chloroplasts still remains unresolved, NO-induced signals directly impact the Fe homeostasis. It seems very likely that the regulatory role of plastidial Fe content on Fe acquisition by chloroplasts (Solti *et al.*, 2012) could also rely on a nitrosative signal. In accordance, the activity of FRO7, a key component in the reduction-based Fe acquisition mechanism of chloroplasts, is suppressed by a slight excess of Fe (Sági-Kazár *et al.*, 2021). Nevertheless, data on the effect of the nitrosative signal on cellular Fe homeostasis still remains controversial, that is, the nitrosative signal seems to be a positive regulator of organellar Fe allocation and Fe storage under optimal Fe nutrition, whereas under Fe deficiency or excess, it is suggested to limit at least Fe allocation to the chloroplasts. In plastids, Fe–S clusters may also be connected with signalling processes. Fe–S cluster biosynthesis is supposedly involved in the feedback suppression of plastidial Fe uptake. The NAP1–NAP7–NAP6 complex, a component of the SUF system, is considered to provide a signal of the Fe status of plastids (for review, see Briat *et al.*, 2007).

It has also been long debated whether aconitase (ACO) or any other Fe–S cluster redox enzyme fulfils a sensor role in plants. For comparison, the cytoplasmic 4Fe–4S redox enzyme ACO monitors the cytoplasmic availability of Fe–S clusters and regulates the expression of *FER* and *Transferrin* genes in human cells (Hernández-Gallardo and Missirlis, 2020; Garza

et al., 2020; Senoura *et al.*, 2020). Based on the *aco1-3* mutant, Arnaud *et al.* (2007) indicated that the regulation of Fe homeostasis by ACO is unlikely in Arabidopsis. In contrast, Senoura *et al.* (2020) reported that rice ACO1 binds RNA and performs upstream regulation of the Fe acquisition transcription factors. Therefore, the Fe signalling mechanisms seem to be taxon specific, and further investigations are required to elucidate the impact of plant ACO on Fe homeostasis across angiosperms.

Intracellular Fe status is in a dynamic and reciprocal interaction with epigenetic regulation, which is involved in the establishment of the complexity of responses to altered Fe nutrition (Shafiq *et al.*, 2020). DNA methylation regulates transcriptional activity (Harris *et al.*, 2018). Sun *et al.* (2021) showed that DNA methylation is important in the induction of Fe-deficiency responses in the roots. Asymmetric DNA methylation of the plant genome is guided by non-coding RNAs (Zhang *et al.*, 2018), the expression pattern of which alters under Fe deprivation (Wang *et al.*, 2021). Although in animal models, the Fe sensitivity of DNA demethylation is also reported (Wang *et al.*, 2018; Camarena *et al.*, 2021), such a mechanism has not yet been reported in plants. As well as DNA methylation, histone modifications, namely acetylation and methylation, are involved in the regulation of Fe homeostasis (Fig. 2). Histone methylation is a repressive mark placed by the Polycomb Repressive Complex 2 (PRC2). The histone methylation pattern is altered under Fe excess in roots; the repressive marks arginine dimethylation H4Arg3me2 and lysine trimethylation H3Lys27me3 induced the suppression of the Fe acquisition system (Séré and Martin, 2020). In the shoot, under conditions of Fe excess, PRC2 targets *YSL1* and *IRON MAN1*, both of which are involved in long-distance Fe signalling (Kumar *et al.*, 2017; Grillet *et al.*, 2018). Although the information on the effect of epigenetic changes on Fe homeostasis in leaf cells is limited, suppressing the function of PRC2 affects the expression of both *FRO7* (positively) and *BTSL1* (negatively under Fe deficiency; Park *et al.*, 2020). Trimethylation of H3Lys4 is also essential in the regulation of Fe-deficiency responses. *FER1*, *FER3*, and *FER14* have promoter H3Lys4me3 signs under Fe excess (Tissot *et al.*, 2019), although the placing of this signal is regulated by as yet unknown processes. Since the ferritin-based Fe storage is of primary importance under multiple physiological conditions, epigenetic regulation of the expression of *FER* genes represents an important, superior regulation of Fe homeostasis as well as the amount of Fe that can accumulate in the leaf cells. In contrast to methylation, histone acetylation makes the DNA chains better available. The histone acetyltransferase enzyme General Control Nonrepressed 5 was shown to be involved in H3Lys9 and H3Lys14 acetylation at the *FRD3* site (Xing *et al.*, 2015); thus, histone acetylation affects root-to-shoot Fe translocation. Although different histone modification mechanisms are known to affect both signalling and effector elements of root Fe homeostasis, information is limited in relation to leaves. Nevertheless, available data and evidence from animal/yeast cells suggest that cellular Fe nutrition generally provokes the alteration of epigenetic markers.

Alteration of foliar iron homeostasis during leaf senescence

The generative processes of monocarpic plants lead to alterations in Fe allocation (Martínez-Ballesta *et al.*, 2020). During leaf senescence, a significant amount of leaf Fe content can be redistributed towards the shoot apex (Zhang *et al.*, 1995), although the extent to which this occurs is rather taxon specific. The re-translocation of Fe on a source–sink basis from leaves towards developing tissues should operate on a symplastic/phloem translocation pathway. In consequence, the biosynthesis of NA is enhanced in leaves during senescence (Shi *et al.*, 2012). Since the degradation of the photosynthetic apparatus and chloroplasts is an important process during leaf senescence (Domínguez and Cejudo, 2021), a controlled dismounting of Fe cofactors from the photosynthetic machinery is required. In parallel with this process, Fe-storing ferritin appears in plastids (Barton, 1970; Fig. 3). Autophagy is an important process in the remodelling of tissues and cell contents. Recent data suggest that vacuolar autophagy of chloroplasts (chlorophagy) is essential in Fe relocation (Pottier *et al.*, 2019). In the autophagy *atg5-1* mutant, Fe translocation from vegetative to generative tissues is reduced (Mari *et al.*, 2020). Based on a comparison with other autophagy-defective mutants, a two-step re-translocation model was suggested where Fe first accumulates in vegetative organs and subsequently remobilizes to seeds (Pottier *et al.*, 2019). Nevertheless, autophagy processes should be accompanied by Fe relocation within the cells, with Fe liberated from the photosynthetic apparatus being accumulated at least temporarily in lytic vacuoles. In consequence, the degradation of the photosynthetic apparatus serves as a source of Fe for re-translocation towards sink tissues from senescing leaves; however, a better understanding of intracellular Fe relocation processes is still required.

Concluding remarks

In mesophyll cells, the transport and homeostasis of Fe is dominated by the proper incorporation of Fe into the photosynthetic electron transport chain. Although in the past 15 years multiple aspects of plastidial Fe homeostasis have been revealed, there is less in the literature concerning the mitochondria of photosynthetically active cells, and thus information on mitochondrial Fe homeostasis is mainly based on root cells. The loading of Fe into redox-active sites has a danger of uncontrolled Fenton reactions; thus, the proper complexing of Fe is essential. Fe–S clusters are among the most important Fe-containing cofactors. Indeed, further investigations are required on their transport across organellar membranes in

mesophyll cells to reveal whether, and to what extent, the organellar Fe–S clusters contribute to the formation of cytoplasmic holoproteins. In the control of cellular and organellar Fe homeostasis, Fe-sensing mechanisms have a pivotal role. Although hemerythrin-domain Fe sensing exists in foliar cells, the understanding of intracellular, and especially organellar, Fe status is far from complete. The amount of data on the connection between epigenetic markers and the Fe status is increasing. However, the mechanisms that lead to the alteration of the epigenetic signs have not been revealed yet. The processing of Fe cofactors in photosynthetically active cells share similarities between mitochondria and plastids, but the coordination of mitochondrial and plastidial Fe homeostasis has not been resolved yet. Finally, Fe homeostasis in leaves undergoes an alteration during senescence, when the leaf status changes from a sink to a source of Fe for developing and generative tissues. Nevertheless, little is known about the intracellular processing of Fe during senescence processes, including the liberation of Fe from the cofactors and the alterations of intracellular Fe transport. Since Fe remobilization from leaves affects both plant growth and crop yield and also the holistic effectiveness of foliar Fe treatment, revealing these intracellular processes will have a high impact in the future.

Conflict of interest

The authors declare no conflict of interest.

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