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► To cite this version:

Stéphane Mari, Christophe Bailly, Sébastien Thomine. Handing off iron to the next generation: how does it get into seeds and what for?. *Biochemical Journal*, 2020, 477 (1), pp.259–274. 10.1042/BCJ20190188 . hal-02445553

HAL Id: hal-02445553

<https://hal.science/hal-02445553>

Submitted on 14 Dec 2020

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title

Handing off iron to the next generation: how does it get into seeds and what for?

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Abstract

To ensure the success of the new generation in annual species, the mother plant transfers a large proportion of the nutrients it has accumulated during its vegetative life to the next generation through its seeds. Iron (Fe) is required in large amounts to provide the energy and redox power to sustain seedling growth. However, free Fe is highly toxic as it leads to the generation of reactive oxygen species. Fe must therefore be tightly bound to chelating molecules to allow seed survival for long periods of time without oxidative damage. Nevertheless, when conditions are favorable, the seed Fe stores have to be readily remobilized to achieve the transition towards active photosynthesis before the seedling becomes able to take up Fe from the environment. This is likely critical for the vigor of the young plant. Seeds constitute an important dietary source of Fe, which is essential for human health. Understanding the mechanisms of Fe storage in seeds is key to improve their Fe content and availability in order to fight the Fe deficiency. Seed longevity, germination efficiency and seedling vigor are also important traits that may be affected by the chemical form under which Fe is stored. In this review, we summarize the current knowledge on seed Fe loading during development, long-term storage and remobilization upon germination. We highlight how this knowledge may help seed Fe biofortification and discuss how Fe storage may affect seed quality and germination efficiency.

Introduction

Seed is a very special stage in the life of a plant. It is the basis for the next generation and the survival of the species. To ensure the success of the new generation, the mother plant transfers a large proportion of the nutrients it has accumulated during its vegetative life to its seeds. Most work on seed nutrient storage focuses on carbohydrate, lipids and proteins, which provide the bulk energy, carbon and nitrogen required to initiate development. Depending on species, 30 to 90% of plant nitrogen is allocated to seeds at the end of the plant life cycle¹. However, mineral stores are also very important to sustain seedling growth. Especially, iron (Fe) is required in large amount in mitochondria, which provide the energy and redox power during the initial stage of seedling development. For example, in the case of *Arabidopsis* (*Arabidopsis thaliana*), 50 to 55% of the iron (Fe) is allocated to seeds at the end of the life cycle^{2,3}. Moreover, the nutrients need to be stored in a compact and stable form. Seed storage proteins form pseudo crystalline aggregates in protein bodies within seed embryo cells. Free Fe is highly toxic as it leads to the generation of reactive oxygen species through the Fenton reaction. In cells, Fe must therefore be tightly bound to chelating molecules to prevent the formation of ROS. This is probably even more important in seeds than in other organs, as seeds represent resistance forms designed to survive for long periods of time, often several years, and through stresses. Nevertheless, when conditions are favorable, the nutrients stored in seeds have to be readily remobilized to sustain the early development of the seedling before it acquires photosynthetic capacity and becomes able to take up nutrients from the environment. Iron is a major component of photosystem I and its availability to the young seedling is necessary to achieve the transition towards active photosynthesis. This is likely critical for the vigor of the young plant.

From the point of view of animals and especially human beings, seeds are a major food resource. They provide carbohydrates, proteins and fat but also micronutrients such as Fe and zinc, which are essential for human health. About 2 billion human beings suffer from Fe deficiency, which leads to asthenia, anemia and in extreme cases to death. The prevalence of Fe deficiency is highest in populations that rely mostly on a plant based diet because plants and seeds in particular do not provide sufficient Fe⁴. Understanding the mechanisms of Fe storage in seeds is key to improve their Fe content and availability in order to fight Fe deficiency using biofortification. From the agricultural perspective, seed longevity, germination efficiency and seedling vigor are also very important traits that breeders and farmers seek to improve. Here also, seed nutrient content and especially the content and

chemical form of Fe may be critical. In this review, we will summarize the current knowledge on seed Fe loading during development, long-term storage and remobilization upon germination, updating the last review on the topic ⁵. We will highlight how this knowledge may help seed Fe biofortification, longevity, germination efficiency and stand establishment.

Iron uptake and translocation to the shoots

Plants take up iron using distinct strategies according to species ^{6, 7}. Most plant species use strategy I, which is based on the reduction of Fe in the rhizosphere followed by its uptake by a transporter for Fe(II). In contrast, graminaceous species, which include the major grain crops such as rice, wheat and barley, have evolved strategy II. Strategy II is based on the release of small Fe(III) chelating molecules called phytosiderophores in the rhizosphere and subsequent uptake of siderophore-Fe(III) complexes. Under Fe deficiency, strategy I plants activate the expression of the genes involved in Fe solubilization and uptake. The molecular players in this response have been identified in Arabidopsis but are essentially conserved in other species. The proton pump Arabidopsis plasma membrane H⁺-ATPase 2 (AHA2) drives rhizosphere acidification which is important to solubilize Fe ⁸. The coumarin transporter Pleiotropic Drug Resistance 9 (PDR9) allows the secretion of coumarin with iron binding and reducing properties in the rhizosphere. Enzymes of the coumarin biosynthetic pathway are induced along with *PDR9* ^{9, 10}. The membrane bound Ferric chelate Reductase FRO2 reduces chelate-bound Fe(III) to Fe(II), which is taken up into root cells by the divalent metal Iron Regulated Transporter 1 (IRT1) ^{11, 12}. IRT1 is not specific for Fe(II) and drives the uptake of a range of other divalent metal cations including Zn, Mn, Co, Cd, Ni ¹³⁻¹⁵. A sophisticated mechanism enables the removal of IRT1 from the plasma membrane when intracellular concentrations of non-Fe metals raise ¹⁶. In addition, several genes involved in the sequestration of these metal cations are activated under Fe deficiency ¹⁷. In graminaceous species such as rice, maize and barley, the components that are upregulated for Fe acquisition under Fe deficiency include the phytosiderophore efflux and Fe-siderophore influx transporters as well as the biosynthetic pathway of phytosiderophores of the mugineic acid family ⁷. The S-adenosyl methionine cycle is activated to produce methionine, which is used by the nicotianamine synthase (NAS) to produce nicotianamine (NA) the precursor of mugineic acids. Whereas NA is also synthesized in strategy I species, mugineic acids are unique to graminaceous species. NA is converted to mugineic acid by the nicotianamine aminotransferase (NAAT) and to deoxymugineic acid by the deoxymugineic acid synthase (DMAS). MA and DMA are secreted to the rhizosphere by an efflux transporter, Transporter

Of Mugineic acid 1 (TOM1) in rice (*Oryza sativa*), Yellow Stripe 3 (YS3) in maize (*Zea mays*)^{18, 19}. Fe-siderophore complexes are taken up by specific influx transporters, Yellow Stripe 1 (YS1) in maize and YS1-Like 15 (YSL15) in rice²⁰⁻²². Despite the difference in their uptake strategies, the control of Fe deficiency responses involves a similar network of negatively and positively regulating bHLH (basic Helix Loop Helix) in graminaceous plants and the other species^{23, 24}. In addition, Hemerythrin Domain containing RING proteins that act as negative regulators of Fe deficiency responses by mediating the Fe dependent degradation of specific bHLH, are also conserved among strategy I and strategy II plants²⁵⁻²⁷. To be translocated to the shoots, Fe has to go through the endodermis cell layer and to be released into the xylem sap together with ligands that maintain it in soluble form. Recently, suberin deposition around the endodermis has been shown to provide a control point for Fe translocation. Under Fe deficiency, the suberin layer is degraded allowing the transfer of Fe from the root cortex to the central cylinder where it can be loaded into the xylem for translocation to the aerial parts^{28, 29}. Based on its expression pattern and plasma membrane localization, the Fe efflux transporter Ferroportin (FPN1)/Iron REGulated 1 (IREG1) has been proposed to mediate Fe loading in the xylem in Arabidopsis³⁰. The MATE family citrate efflux transporters, Ferric Reductase Deficient 3 (FRD3) in Arabidopsis and FRD3- Like 1 (FRDL1) in rice, play an important role in the translocation of Fe and other metals, by loading citrate which chelates Fe and prevent its precipitation in the xylem sap³¹⁻³⁵.

Remobilization from other organs to the seeds

At the vegetative stage, most of the iron translocated to aerial parts is used for photosynthesis in chloroplasts in mesophyll cells. Photosystem I and Ferredoxin, which are very abundant in chloroplasts, contain numerous FeS clusters. This assumption based on biochemical needs is supported by cell fractionation experiments and Fe imaging in leaves³⁶⁻³⁸. Upon transition to the reproductive stage, a large part of the Fe present in vegetative tissues is transferred to the seeds. In Arabidopsis, 50 to 55% of total plant Fe ends up in the seeds at the end of the life cycle (the Iron Harvest Index, **Figure 1**)^{2, 3}. A recent study based on Fe isotope labeling indicated that most of the Fe stored in seeds originates from vegetative tissue, implying a major remobilization of Fe².

Prior to its movement from the vegetative tissues to the seeds, Fe has to be made available in the senescing tissues. Autophagy was proposed to play a key role in nutrient remobilization from vegetative tissues to seeds³⁹. This was based on the finding that autophagy is involved in nitrogen remobilization to seeds⁴⁰. Recently, the finding that a mutation in *Autophagy 5*

(*ATG5*), which strongly impairs autophagy, decreases the proportion of Fe allocated to seeds from 50-55% to 15-20% highlighted the importance of autophagy for Fe remobilization². Similar results were obtained for other micronutrients such as Zn and Mn. The role of autophagy is thus clearly not limited to Fe. Recently, several pathways have been implicated in the degradation of chloroplasts, which contain most of the cellular Fe, during leaf senescence⁴¹. They include (i) the senescence-associated vacuoles (SAV) that are important for the degradation of stromal proteins such as RuBisCo and glutamine synthase, (ii) chlorophagy in which whole chloroplast are encapsulated in autophagosomes⁴² (iii) a vesicular pathway depending on the ATG8 Interacting protein (ATI) which degrades envelope and thylakoid membranes in addition to stromal proteins⁴³ and (iv) CV-Containing vesicles (CCVs) that contain the CV protein (Chloroplast Vesiculation) responsible for chloroplast destabilization⁴⁴. In contrast to the others, the CCV pathway does not require autophagy. It will be interesting to determine the respective importance of these pathways for Fe remobilization during vegetative organ senescence, as they could allow to target some nutrients more specifically than autophagy.

In addition to autophagy, the timing of senescence is also an important factor controlling remobilization. If senescence occurs too fast, the time window for remobilization is reduced and less nutrients are allocated to seeds. Hence, NAC transcription factors, which control the onset of senescence, also play a major role in the control of nutrient remobilization from leaves to seeds⁴⁵⁻⁴⁷. More work will be required to understand how the interplay between autophagy and senescence controls Fe allocation to seeds in different species.

After it has been made available in vegetative tissue, Fe has to be transported to developing seeds through the phloem. Many lines of evidence have pointed to a key role of NA in this process. Complete loss of NA synthesis leads to sterility⁴⁸⁻⁵⁰. This has initially prevented a direct analysis of the role of NA in Fe transport to seeds, as the mutants did not set seeds. The first evidence that NA is important for Fe transport to the seeds came from the study of mutants impaired in *YSL-like* genes (*YSL*). The strong expression of *OsYSL2* in phloem tissues of rice aerial parts and developing seeds and the ability of this transporter to take up NA-Fe and NA-Mn complexes when expressed in *Xenopus* oocytes provided the first indication that NA is important for Fe transport through the phloem to the seeds⁵¹. Analysis of rice lines in which *OsYSL2* expression was silenced further supported this hypothesis⁵². In these lines, Fe transport to the shoots and the seeds was strongly decreased. Conversely, increased *OsYSL2* expression under a phloem specific promoter led to strong increase in seed endosperm Fe concentration⁵². More recently, another YSL transporter from rice, *OsYSL9*, was implicated

in Fe delivery to developing rice grain. *OsYSL9* expression is upregulated by Fe deficiency in roots but down regulated in leaves under the same conditions⁵³. The role of YSL in metal transport to seeds is not restricted to rice. Similar conclusions were obtained from the analysis of Arabidopsis knockout mutants targeting *AtYSL1* gene or double mutants affecting *AtYSL1* and *AtYSL3*⁵⁴⁻⁵⁶. Like *OsYSL2*, *AtYSL1* is expressed in phloem tissues of leaves as well as in developing siliques. Similar to *OsYSL9*, it is down regulated under Fe deficiency. Loss of function mutants in *AtYSL1* accumulated higher levels of NA in shoots but strongly decreased NA and Fe concentrations in mature seeds⁵⁵. Combining a mutation in *AtYSL1* with a mutation in another Arabidopsis YSL strongly expressed in phloem, *AtYSL3*, leads to more severe phenotypes: the *ysl1ysl3* mutants display symptoms of strong Fe deficiency with interveinal chlorosis as well as decreased fertility. Like *ysl1*, they also accumulate lower concentrations of Fe in their seeds when provided with a high Fe concentration in the medium, which alleviates the defect in fertility and up-regulate *YSL1* expression^{55, 56}. Further inflorescence grafting experiments showed that expression of *AtYSL1* and *AtYSL3* in the rosette was sufficient to restore fertility but not seed Fe content⁵⁴. Furthermore, the identification of a quadruple mutant combining knockout in 3 out of the 4 genes encoding NAS in Arabidopsis and a knockdown in the fourth one allowed to obtain fertile plants with drastically decreased levels of NA⁴⁸. In this quadruple *nas* mutant, Fe and NA levels are lower in seeds, similar to the phenotype observed in *ysl1*. Interestingly, in NA biosynthesis mutants, Fe accumulates in phloem cells indicating that NA is required to retrieve Fe from the phloem and provide it to sink tissues, such as seeds⁵⁷. This role may be shared between NA and mugineic acids in graminaceous plants. On the other hand, all YSL transporters characterized so far using expression in yeast or in *Xenopus* oocytes mediate the influx of NA-Fe or MA-Fe complexes into cells. The finding that NA and YSL are important for phloem transport of Fe does not imply that Fe is transported as NA-Fe complex in the phloem. Another important player in Fe transport from vegetative organs to the seeds is the oligopeptide transporter OligoPeptide Transporter 3 (OPT3). Full loss of function of *OPT3* leads to embryo lethality suggesting that the substrate of OPT3 is an essential cellular metabolite⁵⁸. Even though the closest homologue of OPT3, BjGT1, is a glutathione transporter⁵⁹, transport assays in yeast failed to show OPT3 ability to transport GSH⁶⁰. Instead, expression of *OPT3* could complement the yeast *fet3fet4* mutant impaired in Fe uptake, suggesting that it transports Fe complexes. The exact substrate of OPT3 remains to be identified. The identification of a weak allele of *opt3*, *opt3-2* allowed investigating its function at the reproductive stage⁶¹. In *opt3-2* mutants, Fe concentration is increased in

vegetative organs but decreased in seeds, indicating a role in Fe transfer from vegetative organs to seeds. More recently, OPT3 was shown to be targeted to the plasma membrane and mainly expressed in phloem cells^{60, 62}. In *opt3-2* mutants, Fe concentration was strongly increased in the xylem sap and decreased in the phloem sap, pointing to role of OPT3 in Fe transfer from the xylem to the phloem⁶⁰. Combined with its expression in phloem cells, this suggests that OPT3 is involved in Fe loading into the phloem. Hence, both OPT3 and NA play critical roles in long distance transport of Fe in the phloem to sink organs and especially seeds: OPT3 would be necessary for loading Fe into the phloem, while NA would be required for Fe retrieval from the phloem^{57, 60}. Interestingly, OPT3 is also important for Fe deficiency signaling and *opt3-2* up-regulated Fe-deficiency responsive gene expression even under Fe sufficient conditions^{60, 62}. In rice, OsOPT7, a homologue of OPT3, was characterized⁶³. OsOPT7 could not transport GSH or Fe when expressed in *Xenopus* oocytes and yeast. Like *AtOPT3*, *OsOPT7* is induced under Fe deficiency but its expression is more widespread than that of *OPT3*^{61, 63}. Moreover, the phenotype of *opt7* mutant does not resemble that of *opt3-2*. Whether graminaceous plant genomes carry orthologues of OPT3 remains to be determined.

Iron loading into seed tissues

There is no continuity between the vasculature of the mother plant and the pro-vasculature of the embryo. Therefore, Fe, as other nutrients, has to be released from the phloem and re absorbed by the embryo. In *Arabidopsis* and other dicotyledonous plants, nutrients are released at the level of the chalaze and the nucellus into the embryo sac fluid from which the embryo takes up nutrients (Figure 2). Accordingly, *AtYSL1* expression is detected in the funiculus and the chalazal endosperm⁵⁵. Analyses of Fe speciation in the embryo sac fluid of developing pea seeds revealed that Fe is present as ferric Fe bound to citrate and malate in this extracellular compartment⁶⁴. In agreement with this finding, the citrate efflux transporter *FRD3* is expressed in the peripheral cell layer of the embryo and the cell layer of the tegument facing the embryo sac during seed development (Figure 2)³⁴. This suggest that *FRD3* secretes citrate in the embryo sac to maintain Fe solubility and availability for uptake by the embryo. In dicotyledonous plants, Fe is taken up as ferrous Fe. This implies that Fe must be reduced prior to uptake by the embryo. However, genetic analyses in *Arabidopsis* failed to identify a membrane bound ferric chelate reductase that is important for Fe acquisition by the embryo among *FRO2* homologues. Instead, further analysis of the embryo sac fluid in pea showed the presence of a high concentration of ascorbate, sufficient to reduce Fe(III) to Fe(II) prior to its uptake by the embryo⁶⁴. Consistently, in *Arabidopsis vtc* (*vitamin c*) mutants deficient in

ascorbate biosynthesis, the seed Fe content is decreased. The speciation of Fe in the embryo sac is thus similar to that encountered in other extracellular compartments of the plant, such as the xylem sap. In contrast, it is striking that ascorbate is used for Fe reduction, whereas membrane bound ferric chelate reductases are used for uptake in roots and leaves as well as in intracellular organelles such as mitochondria and plastids^{11, 65, 66}. The transporters responsible for secreting ascorbate and Fe in the embryo sac remain to be identified. In the case of Zn, the Heavy Metal pumping P-type ATPases HMA2 and HMA4 release Zn from the mother tissues for subsequent uptake by the embryo⁶⁷. In the case of Fe, the plasma membrane Fe efflux transporter IREG1/FPN1 may be involved in this process but so far no defect in Fe supply to the embryo has been reported for *ireg1/fpn1* mutant³⁰.

In wheat and barley, the nutrients, including Fe, are provided to the grain by a single vascular strand along the ventral crease⁶⁸. Fe is mostly provided by the phloem and moves through several specialized cell layers: the crease vascular parenchyma, the pigment strand and the nucellar projection. Fe ends up in the transfer cells that are facing the embryo. From there, Fe has to be released in the extracellular space separating the mother plant from the embryo (Figure 3). The transfer cells, as the modified aleurone cells facing them on the side of the embryo, have highly invaginated plasma membranes favoring nutrient release and reabsorption⁶⁸. Fe accumulation and speciation in these structures show marked contrasts in the mature wheat grain. Fe is highly concentrated in the nucellar projection and co-localizes with sulfur. Fe accumulates to a lesser extent in the modified aleurone, from where it is probably distributed to the other aleurone cells, the embryo and the endosperm⁶⁹. X-ray absorption spectra indicate that in the nucellar projection, Fe is mostly associated with NA, whereas in the modified aleurone it is associated with phytate⁶⁹. In agreement with the speciation, barley homologues of AtVIT1, which drives Fe influx into the vacuole where phytate is localized, are strongly expressed in the aleurone^{68, 70-72}. In contrast, the genes encoding NAAT, NAS and YSL, that favor Fe mobility, are expressed at high levels in transfer cells^{68, 73}(Figure 3). In the future, it will be important to determine the specific expression pattern and the role of each of the genes involved in Fe transport in this complex structure. This should allow the identification of the key transporters that release Fe to the extracellular space from the transfer cells as well as those responsible for taking it up into the embryo in the modified aleurone cells.

Iron storage in seeds

The localization of Fe in seeds differs according to species and seed developmental stage. The localization and subcellular localization are strongly associated with Fe speciation, i.e. the nature of the ligand that binds Fe and determines its bioavailability⁷⁴. For example, Fe phytate complexes that are stored in vacuoles are notoriously poorly bioavailable⁷⁵. In contrast, ferritin Fe stored in plastids constitutes a highly bioavailable source of Fe⁷⁶.

Iron and other metals are not distributed evenly in seed tissues. In contrast, Fe distribution follows striking patterns. For example, in *Arabidopsis* mature embryo, Fe is highly concentrated around the vascular tissues^{71, 77-81}. Interestingly, the pattern of Fe distribution is distinct from that of other metals: Mn is concentrated in the sub-epidermal cell layers of *Arabidopsis* cotyledons in mature seeds while Zn is evenly distributed in *Arabidopsis* embryo^{71, 77, 78, 81}. The patterns of metal localization were initially discovered using synchrotron X-ray Fluorescence imaging of intact seeds and were then confirmed and refined using additional approaches. The use of Perls staining intensified by Diaminobenzidine (Perls DAB) allowed identification of the precise cell type accumulating Fe as the proto endodermis of the embryo⁸⁰. The use of micro Particle Induced X-ray Emission (μ PIXE) allowed quantification of the pattern: even though it is concentrated in proto-endodermal cells, Fe is present in other cells, albeit at much lower concentrations, and the Fe concentrated around pro-vascular tissues accounts for 50% of total seed Fe⁸¹. Finally, the use of Electron Dispersive X-ray imaging coupled to Transmission Electron Microscopy (TEM EDX) provided higher spatial resolution allowing the determination of the subcellular localization of Fe in the globoids of vacuoles of proto-endodermal cells (Figure 4)^{79, 82}. This subcellular localization is in agreement with the finding that mutations in AtVIT1 (Vacuolar Iron Transporter 1) disrupt the pattern of Fe distribution^{71, 79}. In *vit1* mutants, Fe is not concentrated around vascular tissues but instead co-localizes with Mn in sub-epidermal cells^{71, 79}. Nevertheless, Fe is still localized in vacuoles in *vit1* mutants⁷⁹. Recently, the vacuolar Mn transporter AtMTP8 (Metal Tolerance Protein 8) was shown to be responsible for concentrating Mn in sub-epidermal cells in wild type seeds and Fe localization in these cells in the *vit1* mutant background^{77, 78}. In *mtp8* knockout mutant seeds, Mn is concentrated around the vascular tissues together with Fe. In a double mutant combining *vit1* and *mtp8* mutations, Mn and Fe are evenly distributed in *Arabidopsis* embryo⁷⁸. Therefore, by creating strong sinks for Fe or Mn, vacuolar metal transporters determine the pattern of metal distribution in mature *Arabidopsis* embryo. The phenotype of *vit1* mutant, which survives poorly when germinated under Fe-deficient conditions, indicates that adequate tissue localization in the embryo is crucial^{71, 79}. Little information is available on how the Fe distribution pattern is generated during embryo

development. Consistent with a strong expression of VIT1 during seed development, Fe patterning is already apparent at the torpedo stage at the center of the cotyledons where the provascular tissue will differentiate^{71, 80}. However, in *Brassica napus*, high resolution Perls DAB imaging detects Fe in the nuclei of all embryo cells at the torpedo stage⁸³. Further during development, at the bent cotyledon stage, Fe is detected in cytoplasmic vesicles and finally ends up in the vacuoles of the cells surrounding the vasculature in mature embryo⁸³. The observation of Fe in the nucleus at the torpedo stage in *B. napus* is consistent with the detection of high Fe concentrations in the nucleus of embryo cells in developing pea (*Pisum sativum*) seeds⁸⁴. Therefore, Fe subcellular localization evolves during embryo development. These changes in localization are most likely paralleled by changes in speciation and therefore of Fe bioavailability. The analysis of Fe bioavailability in pea seeds at different developmental stages show that Fe bioavailability in immature peas is higher than in mature pea seeds⁸⁵.

Whether the pattern of Fe distribution in the vacuoles of cells surrounding the vasculature described in Arabidopsis is conserved among angiosperm has been investigated. In beans (*Phaseolus vulgaris*), Fe stores also concentrates around vascular tissues⁸⁶. Recently, this observation was extended to Brassicales and even Rosids, which represent a wide group including a third of the angiosperms^{87, 88}. However, in many cases the number of cell layers that accumulate Fe is increased, to 2, the endodermis and one cortical cell layer in Brassicales, or even more cortical cell layers in other Rosids, compared to endodermis only in Arabidopsis^{83, 87, 88}. While screening representatives of the different groups of angiosperm, interesting exceptions were identified in *Chenopodium quinoa* and *Carica papaya*^{87, 88}. There are certainly more diverse patterns to be discovered.

In grains, the seeds of graminaceous species, the distribution of Fe is very different from that observed in Brassicales. In grains, in contrast to Arabidopsis, the embryo represents only a small volume of the seed, while the major part is constituted by the starchy endosperm. The endosperm is surrounded by the so called aleurone cell layer. During germination, the aleurone layer is activated by gibberellins, releases enzymes that digest the carbohydrates stored in the endosperm and undergoes programmed cell death^{89, 90}. The embryo is equipped with an absorptive structure called the scutellum, somewhat equivalent to human placenta, which take up the nutrients from the endosperm. In grains, a large part of the Fe is concentrated in the aleurone layer and another pool is found in the embryo^{69, 91, 92}(Figure 3). In wheat (*Triticum durum*), the highest concentrations of Fe are found in the aleurone layer and in the scutellum⁹². Separate measurements of wheat flour and bran indicated that almost

60% of grain Fe is in the bran which includes the aleurone layer and therefore represents the major Fe pool in grains⁹³. Within the aleurone layer, Fe is concentrated together with phosphorus and other minerals in globoids inside the protein storage vacuole, similar to the subcellular localization observed in *Arabidopsis*⁷². In situ X-ray absorption spectroscopy analyses have indicated that in the aleurone layer, most Fe is bound to phytate, either as Fe(II) or Fe(III) in good agreement with its subcellular localization^{69, 92}. Another pool is bound to citrate, which may be involved in the transport from modified aleurone cells present in the crease to aleurone cells⁶⁹. In rice, Fe accumulates in the aleurone layer together with phytate and in the scutellum, as observed in wheat^{91, 94}. The exact speciation of Fe in the embryo and in the endosperm has not yet been determined in these tissues that contain lower Fe concentration, probably because of limitations in the sensitivity of X-ray absorption spectroscopy. Based on co-occurrence of elements, it has been proposed that Fe is bound to phosphate (possibly as phytate) in the scutellum in wheat and to other ligands in the other parts of the embryo and in the endosperm^{69, 92}. These ligands could include proteins or smaller molecules such as NA, which is present in grains as in seeds of non graminaceous species^{55, 95}. In wheat flour which corresponds to the starchy endosperm, NA is the main ligand for Fe⁹⁶.

Little is known on the mechanisms that control the pattern of Fe localization in grains. A developmental analysis showed that throughout rice grain development Fe co-localizes with phosphorus in the aleurone cell layer, indicating that Fe is most likely bound to phytate as soon as it is stored⁹¹. Mutations in OsVIT1, OsVIT2 and Mitochondrial Iron Transporter (MIT) have been shown to perturb iron localization within the embryo^{97, 98}. OsYSL9 is strongly expressed in the scutellum and participates to Fe storage in the embryo⁵³. The phytosiderophore efflux transporter TOM2 is expressed in the dorsal vascular bundle, epithelium and the scutellum of the embryo and may also participate in the distribution of Fe¹⁹. Silencing TOM2 gene expression did not alter seed total Fe content. Analysis of Fe distribution using sXRF, Perls staining or seed dissection could provide more insights in its function in developing rice grains.

Biofortification

The abundance, the localization and the speciation of Fe are equally important parameters to be taken into account in attempts to increase Fe content and bioavailability in grains, which are two of the major targets of biofortification projects. Having the major Fe pool stored as Fe phytate in the aleurone layer is probably the worst combination for human nutrition, as

phytate has extremely high affinity for metals, which drastically limits Fe absorption in the intestine⁷⁵. Moreover, the aleurone layer is most of the time discarded during grain processing, such as polishing for rice or preparation of white flour for wheat^{93, 96, 99}. Hence, biofortification strategies using targeted molecular approaches have aimed at changing both the localization and the speciation of Fe in grains. Several reviews have been recently published on the attempts made to biofortify wheat and rice grain⁹⁹⁻¹⁰¹. This review will therefore not extensively cover the topic of biofortification and rather just highlight a few examples. The initial attempt to biofortify rice grains through biotechnology used the gene encoding ferritin, which provides Fe under a highly bioavailable form, driven by an endosperm specific promoter¹⁰². Although the work provided a critical proof of concept, the increase in Fe content was modest and variable¹⁰³. Probably the most efficient strategy for biofortification by manipulating a single gene has been the activation or the over expression of NA synthase in rice grains⁹⁵. This resulted not only in a strong increase in grain Fe concentration but also in Fe bioavailability, as NA appears to favor Fe intestinal absorption⁹⁵. More recently, expression of a Fe vacuolar transporter TaVIT2 in wheat endosperm was also shown to be a relevant approach to increase Fe content and bioavailability⁷⁰. Probably, the expression of TaVIT2 in the endosperm vacuoles creates a strong Fe sink during development. Subsequently, Fe becomes available when endosperm cells die and lose their internal structure. Current efforts use pyramiding of several constructs to combine the benefits of multiple transgenes. This way, by combining the expression of transporters that limit vacuolar storage with elevated ferritin and NA synthesis, an increase in Fe concentration in grains by up to 6-fold has been obtained¹⁰⁴.

A role for iron in the regulation of seed germination?

Seed germination starts with water uptake by the dry seed and ends with radicle protrusion and initiation of cell division¹⁰⁵. This is the critical phase of plant emergence because it is tightly regulated by water availability, temperature, oxygen, and light conditions. Germination is also controlled by endogenous factors such as the plant hormones abscisic acid (ABA), gibberellins (GA) and ethylene that play a major role in regulating early seed germination. These endogenous signals regulate germination through the process of dormancy, which is an endogenous block to the completion of germination of a mature seed¹⁰⁶. Germination is followed by seedling growth during which mobilization of reserves and nutrient stored within the seed sustain active cell metabolism till the acquisition of autotrophy. Thus, stand

establishment, the first critical component of crop yield, results from the successful and fast completion of both germination and early seedling growth.

Although the role of iron in post-germinative events is quite well documented (see below) its possible involvement in the regulation of germination, *i.e.* the sum of molecular events that allow radicle protrusion, is rather unknown. The effect of iron on seed germination has mostly been documented in the context of soil toxicity, since high Fe concentrations are toxic and inhibit germination^{107, 108}, but this is a common feature to many metals. Increase in phytate-degrading enzyme activity has been reported to occur during seed germination, with a concomitant decline in phytate¹⁰⁹⁻¹¹¹ thus increasing iron bioavailability¹⁰⁹. Müller et al. (2009) showed that hydroxyl radicals were likely to play a role in cell wall loosening during radicle elongation and weakening of the endosperm of cress seeds¹¹². Although Fenton reaction is a natural candidate mechanism for explaining hydroxyl radical production they did not provide any evidence about its possible involvement *in vivo*. Thus, whether iron availability participates to the numerous biochemical and molecular processes involved in the regulation of radicle protrusion is actually not known.

Murgia and Morandini (2017) demonstrated that, when seeds developed on the mother plant under Fe deficiency, they were more dormant at harvest, thus suggesting that seed Fe availability can regulate *sensu stricto* germination¹¹³. It is highly likely that Fe could regulate germination by controlling ROS homeostasis, through its involvement in the Fenton reaction. ROS indeed have been proposed to be key players in seed germination and dormancy¹¹⁴⁻¹¹⁶. Seed dormancy alleviation requires controlled ROS generation¹¹⁷ but excessive levels of ROS trigger oxidative stress and prevent germination. This is occurring when seeds are germinated in unappropriated environmental conditions or when they have aged¹¹⁵. With regards to the relationship between Fe and ROS metabolism, we can thus hypothesize that Fe homeostasis, as controlled by sequestration and release by vacuoles, is likely to participate in the ability of seeds to germinate and to play a role in the regulation of dormancy, by buffering ROS homeostasis. However whether Fe speciation (and localization) is important for seed longevity and control of dormancy and germination remains to be addressed.

Iron remobilization after germination

Besides human nutrition, the main role of seed Fe stores is to sustain the early development of seedlings after germination. In dicots in which most of the Fe is probably stored as Fe phytate in vacuoles, retrieving Fe stores likely requires first to break down the phytate, then to transport Fe out of the vacuole and out of the cells. In Arabidopsis, Perls DAB staining

allowed monitoring the fate of Fe stores after germination: the high Fe concentration around
 vascular tissues observed in dry seeds disappeared within 4 days, indicating that Fe was
 rapidly redistributed the growing organs of the seedling^{79, 80}. In Arabidopsis, this process
 requires two redundant vacuolar transporters AtNRAMP3 and AtNRAMP4 (Natural
 Resistance Associated Macrophage Protein)⁸². AtNRAMP3 and AtNRAMP4 are highly
 expressed after radicle protrusion. In loss of AtNRAMP3 and AtNRAMP4 function mutants,
 seedling growth is arrested before the onset of photosynthesis⁸². In the *nramp3nramp4*
 double mutant, Fe remains blocked inside the endodermal vacuoles after germination^{79, 80, 82}.
 The phenotype of *nramp3nramp4* is partially suppressed by mutations in AtVIT1 by
 redirecting Fe storage in vacuoles of cortical cells⁷⁹. The phenotype of *nramp3nramp4* is also
 rescued by Fe supplementation. However, even in the presence of Fe in the medium, the
 mutant activates Fe deficiency responses¹¹⁸. Interestingly, chloroplast functions and
 especially Fe-requiring plastidial enzymes are repressed in the mutant while mitochondrial
 function is maintained¹¹⁸. This suggests that Fe is prioritized to mitochondria or that these
 organelles rely on an independent pool of Fe. AtNRAMP3 and AtNRAMP4 encode divalent
 cation transporters. As Fe is most likely stored as Fe(III) in the vacuole, it means that Fe
 reduction is probably required prior to Fe export by AtNRAMP3 and AtNRAMP4. However,
 the Fe reduction system active in vacuoles of germinating cells remains to be identified. The
 phytase allowing Fe release prior to its reduction and export also remains to be identified. The
 importance of such activity is highlighted by the finding that expression of the bacterial
 phytase US417 or mutation in the phytate biosynthetic enzyme IPK1 (Inositol Phosphate
 Kinase 1) in Arabidopsis significantly accelerate Fe remobilization¹¹⁹. After its export from
 the vacuole, Fe needs to be exported from storage cells (the endodermis in Arabidopsis).
 However, the transporters involved in Fe efflux from cells during germination have not been
 identified. One candidate could be IREG1/FPN1, the plant homologue of the transporter
 involved in the release of Fe into the blood flow from intestine epithelial cells³⁰. Once it is
 exported from the cells, Fe needs to remain in a soluble form to diffuse to other cells. The
 citrate efflux transporter FRD3 is expressed in germinating seeds and could be involved in the
 release of citrate to form soluble Fe complexes in the apoplast. This hypothesis is supported
 by the finding that *frd3* mutants are chlorotic and exhibit slow root growth after germination³⁴.
 This phenotype can be rescued by supplementation of Fe or citrate in the cytosol.
 In relation with their distinct organization, Fe remobilization mechanisms in seeds of
 graminaceous species are likely very different. In grains, most of the Fe is in the aleurone
 layer, outside of the embryo. During germination, under the positive control of gibberellins,

aleurone cells secrete amylases that digest the starch in the endosperm and eventually undergo
 programmed cell death (PCD)⁸⁹. Membranes are destroyed in this process and it is likely that
 the content of globoids including Fe bound to phytate is released in the endosperm. Iron
 efflux from vacuoles is probably not a necessary step for mobilization. Accordingly, no
 orthologues of AtNRAMP3 or AtNRAMP4 have been reported in graminaceous plants. In the
 endosperm, phytate is probably hydrolyzed by phytase and the released Fe bound to NA. As
 in dicotyledonous species, the precise phytase involved have not yet been identified. Then, the
 main question is how is Fe taken up from the endosperm into the embryo and further
 distributed to growing organs within the embryo. Time course analysis of gene expression and
 Fe localization during rice germination has highlighted several candidates^{94, 120}. X-ray
 fluorescence imaging revealed that after 24h of germination, Fe accumulates in the epithelium
 and scutellum, the Fe level decreases in the scutellum and increases in the coleoptile. Iron is
 already visible in the root tip after 36 hours⁹⁴. Genes encoding NA and phytosiderophore
 biosynthesis were upregulated during germination¹²⁰. Interestingly, NA synthase expression
 was prominent in the endosperm while NA amino transferase (NAAT) was expressed in the
 embryo. Accordingly, several members of the YSL NA and phytosiderophore transporters,
 YSL2, 6, 10, 12 and 14 are upregulated¹²⁰. The expression of TOM2, a NA and
 phytosiderophore efflux transporter and of OsYSL2 increased markedly in the scutellum
 during germination^{19, 120}. These data indicate that Fe is transported as a complex with NA
 into the embryo or phytosiderophores within the embryo. Several transporters for free divalent
 Fe were also upregulated during germination: OsIRT1, several OsNramp genes and a
 Ferroportin/IREG^{94, 120}. As in dicotyledonous species, the citrate efflux transporter OsFRDL1
 is upregulated indicating that citrate is also secreted to maintain Fe solubility during cell-to-
 cell transport^{34, 35}. Finally, the rice homologue of the chloroplast Fe influx system in
 Arabidopsis PIC1 is upregulated after 3 days probably to sustain the differentiation of plastids
 into chloroplast during early seedling development¹²¹. Many transporters of Fe²⁺ or Fe
 complexes with NA or siderophores are apparently involved in Fe remobilization after grain
 germination. However, the individual contribution of each of these transporters remains to be
 analyzed to identify the key steps in Fe remobilization in graminaceous species.
 With its uptake into chloroplasts of the developing seedling, Fe completes its journey from the
 senescing leaves of the mother plant to the expanding photosynthetic apparatus of its progeny,
 via the seed.

Conclusions and perspectives

Many transporters and ligands for Fe have been characterized and this knowledge has been used to design targeted biofortification strategies. However, many key steps remain to be elucidated at the molecular level. The mechanisms that make iron available for reallocation to the seeds during leaf senescence need to be explored in more details. It would be important to determine which of the different pathways that contribute to chloroplast degradation are most important for Fe mobilization. Important questions also still need to be answered concerning phloem transport: the ligand for Fe in the phloem is still not unequivocally identified. Moreover, whereas OPT3 plays a key role in Fe transport to seeds, the specific substrate of this transporter is still unknown. The transfer of Fe from the mother plant to the embryo has been under investigated so far and probably deserves more attention (Figure 2). The pioneering discovery that ascorbic acid is responsible for Fe reduction in the extracellular space separating the mother tissues from the embryo raises important questions. How general this mechanism is remains to be determined. For example, the relevance of this finding for grain staples needs to be addressed. There is also a need to identify the molecular players involved in ascorbate secretion and regeneration. Moreover, even though FRD3 has been proposed to be responsible for citrate efflux in the intracellular space separating the maternal tissues and the embryo, this hypothesis would need to be substantiated and other transporters potentially involved in citrate and malate efflux need to be identified. Last but not least, the transport mechanisms responsible for Fe efflux from mother tissues and reuptake into the embryo remain completely unknown. Tissue specific transcriptomic analyses have provided candidates for mediating these steps in barley ⁶⁸. The role of each candidate has now to be analyzed in detail and similar analyses are lacking in Arabidopsis or other dicots. Manipulating these steps would certainly open new perspectives for biofortification. Besides the effectors of Fe transport and complexation during Fe loading into seeds, it would also be important to get insights into the master regulators that control Fe storage in seeds. How Fe storage is connected to the signaling pathways and transcription factors that regulate seed development, such as Leafy Cotyledon 2 (LEC2) or WRINCKLED, needs to be unraveled ¹²². When considering Fe remobilization after germination, many questions are still open. In Arabidopsis and in dicots in general, several steps upstream and downstream of the action of vacuolar efflux by NRAMP3/4 remain to be investigated. The molecular identity of the phytases that make Fe available for transport has to be determined. Whether Fe needs to be reduced prior to transport by vacuolar NRAMP and by which mechanism are still open questions. Downstream of vacuolar efflux the plasma membrane transporters that allow Fe efflux from endodermal cells to other cell types, as well as the mechanisms that allow Fe

distribution to plastids, are still to be identified. Similar questions arise concerning the use of Fe stored in aleurone cells after the germination of grains of monocots. Finally, the master regulators that orchestrate Fe remobilization in response to hormonal cues, such as gibberellins, also need to be defined.

In conclusion, even though many players involved in Fe storage and remobilization from seeds have been discovered in the last decades, we still lack a molecular understanding of several key steps. In addition to improving our basic knowledge of how plants transfer nutrients from one generation to the next, unraveling these steps holds great promises for seed Fe biofortification and possibly for improving seed quality and stand establishment.

Acknowledgements

The work on seed iron storage in ST, SM and CB laboratories is supported by the CNRS, the INRA and Paris Sorbonne University, as well as by the collaborative ANR grant ISISTOR (ANR-16-CE20-0019-02).

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Figure 1: Fe uptake and remobilization to seeds in *Arabidopsis thaliana*. The names of the main proteins involved in uptake and translocation from roots to shoots are indicated in blue. The name of the main proteins involved in remobilization to seeds are indicated in brown. The percentages indicate the mean distribution of Fe at the end of the plant's life cycle, excluding the roots (adapted from Pottier et al., 2019)

Figure 2: Fe transfer from the maternal to embryo tissues. A. Fe is transported to the maternal tissues of the developing seed but has to cross an extracellular space between the teguments and the embryo. B. Fe is bound to malate and citrate in the extracellular space separating the maternal tissues from the embryo. In Arabidopsis and pea, Fe is reduced by ascorbate prior to its uptake into the embryo. The transporters responsible for Fe, malate and ascorbate secretion to the extracellular space as well as the transporter responsible for Fe uptake into the embryo remain to be identified.

Figure 3: Fe distribution in a mature cereal grain (eg wheat grain). Fe-rich tissues and cells appear in blue, symplastic disconnections (between maternal tissues and endosperm; between endosperm and scutellum) are highlighted in pink. The size, shape and development of the crease and the nucellar projection vary among the monocot phylogeny.

Figure 4: Fe distribution in *Arabidopsis thaliana* mature embryo. A. Perl's DAB staining of Fe in the whole embryo shows high concentration of Fe (brown color) around the pro-vasculature. B. TEM micrograph of an embryo section in the cotyledon showing the pro-vasculature (pv), proto-endodermis cells (en) and subepidermal cells (se). The phytate globoids appear as small black dots inside the storage vacuole (arrow). C. EDX analysis of protein bodies in proto-endodermal (en) or sub-epidermal cells (se): Fe is concentrated in the storage vacuole of proto-endodermal cells (en).