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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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1 **title**

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

4

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6

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15

16 **Abstract**

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

34
35

Introduction

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

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

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

72

73 **Iron uptake and translocation to the shoots**

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

102 Of Mugineic acid 1 (TOM1) in rice (*Oryza sativa*), Yellow Stripe 3 (YS3) in maize (*Zea*
103 *mais*)^{18, 19}. Fe-siderophore complexes are taken up by specific influx transporters, Yellow
104 Stripe 1 (YS1) in maize and YS1-Like 15 (YSL15) in rice²⁰⁻²². Despite the difference in their
105 uptake strategies, the control of Fe deficiency responses involves a similar network of
106 negatively and positively regulating bHLH (basic Helix Loop Helix) in graminaceous plants
107 and the other species^{23, 24}. In addition, Hemerythrin Domain containing RING proteins that
108 act as negative regulators of Fe deficiency responses by mediating the Fe dependent
109 degradation of specific bHLH, are also conserved among strategy I and strategy II plants²⁵⁻²⁷.
110 To be translocated to the shoots, Fe has to go through the endodermis cell layer and to be
111 released into the xylem sap together with ligands that maintain it in soluble form. Recently,
112 suberin deposition around the endodermis has been shown to provide a control point for Fe
113 translocation. Under Fe deficiency, the suberin layer is degraded allowing the transfer of Fe
114 from the root cortex to the central cylinder where it can be loaded into the xylem for
115 translocation to the aerial parts^{28, 29}. Based on its expression pattern and plasma membrane
116 localization, the Fe efflux transporter Ferroportin (FPN1)/Iron REGulated 1 (IREG1) has been
117 proposed to mediate Fe loading in the xylem in Arabidopsis³⁰. The MATE family citrate
118 efflux transporters, Ferric Reductase Deficient 3 (FRD3) in Arabidopsis and FRD3- Like
119 1(FRD1) in rice, play an important role in the translocation of Fe and other metals, by
120 loading citrate which chelates Fe and prevent its precipitation in the xylem sap³¹⁻³⁵.

121

122 **Remobilization from other organs to the seeds**

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

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

136 (*ATG5*), which strongly impairs autophagy, decreases the proportion of Fe allocated to seeds
137 from 50-55% to 15-20% highlighted the importance of autophagy for Fe remobilization ².
138 Similar results were obtained for other micronutrients such as Zn and Mn. The role of
139 autophagy is thus clearly not limited to Fe. Recently, several pathways have been implicated
140 in the degradation of chloroplasts, which contain most of the cellular Fe, during leaf
141 senescence ⁴¹. They include (i) the senescence-associated vacuoles (SAV) that are important
142 for the degradation of stromal proteins such as RuBisCo and glutamine synthase, (ii)
143 chlorophagy in which whole chloroplast are encapsulated in autophagosomes ⁴² (iii) a
144 vesicular pathway depending on the ATG8 Interacting protein (ATI) which degrades envelope
145 and thylakoid membranes in addition to stromal proteins ⁴³ and (iv) CV-Containing vesicles
146 (CCVs) that contain the CV protein (Chloroplast Vesiculation) responsible for chloroplast
147 destabilization ⁴⁴. In contrast to the others, the CCV pathway does not require autophagy. It
148 will be interesting to determine the respective importance of these pathways for Fe
149 remobilization during vegetative organ senescence, as they could allow to target some
150 nutrients more specifically than autophagy.

151 In addition to autophagy, the timing of senescence is also an important factor controlling
152 remobilization. If senescence occurs too fast, the time window for remobilization is reduced
153 and less nutrients are allocated to seeds. Hence, NAC transcription factors, which control the
154 onset of senescence, also play a major role in the control of nutrient remobilization from
155 leaves to seeds ⁴⁵⁻⁴⁷. More work will be required to understand how the interplay between
156 autophagy and senescence controls Fe allocation to seeds in different species.

157 After it has been made available in vegetative tissue, Fe has to be transported to developing
158 seeds through the phloem. Many lines of evidence have pointed to a key role of NA in this
159 process. Complete loss of NA synthesis leads to sterility ⁴⁸⁻⁵⁰. This has initially prevented a
160 direct analysis of the role of NA in Fe transport to seeds, as the mutants did not set seeds. The
161 first evidence that NA is important for Fe transport to the seeds came from the study of
162 mutants impaired in *YSL-like* genes (*YSL*). The strong expression of *OsYSL2* in phloem tissues
163 of rice aerial parts and developing seeds and the ability of this transporter to take up NA-Fe
164 and NA-Mn complexes when expressed in *Xenopus* oocytes provided the first indication that
165 NA is important for Fe transport through the phloem to the seeds ⁵¹. Analysis of rice lines in
166 which *OsYSL2* expression was silenced further supported this hypothesis ⁵². In these lines, Fe
167 transport to the shoots and the seeds was strongly decreased. Conversely, increased *OsYSL2*
168 expression under a phloem specific promoter led to strong increase in seed endosperm Fe
169 concentration ⁵². More recently, another YSL transporter from rice, *OsYSL9*, was implicated

170 in Fe delivery to developing rice grain. *OsYSL9* expression is upregulated by Fe deficiency in
171 roots but down regulated in leaves under the same conditions ⁵³. The role of YSL in metal
172 transport to seeds is not restricted to rice. Similar conclusions were obtained from the analysis
173 of Arabidopsis knockout mutants targeting *AtYSL1* gene or double mutants affecting *AtYSL1*
174 and *AtYSL3* ⁵⁴⁻⁵⁶. Like *OsYSL2*, *AtYSL1* is expressed in phloem tissues of leaves as well as in
175 developing siliques. Similar to *OsYSL9*, it is down regulated under Fe deficiency. Loss of
176 function mutants in *AtYSL1* accumulated higher levels of NA in shoots but strongly decreased
177 NA and Fe concentrations in mature seeds ⁵⁵. Combining a mutation in *AtYSL1* with a
178 mutation in another Arabidopsis *YSL* strongly expressed in phloem, *AtYSL3*, leads to more
179 severe phenotypes: the *ysl1ysl3* mutants display symptoms of strong Fe deficiency with
180 interveinal chlorosis as well as decreased fertility. Like *ysl1*, they also accumulate lower
181 concentrations of Fe in their seeds when provided with a high Fe concentration in the
182 medium, which alleviates the defect in fertility and up-regulate *YSL1* expression ^{55, 56}. Further
183 inflorescence grafting experiments showed that expression of *AtYSL1* and *AtYSL3* in the
184 rosette was sufficient to restore fertility but not seed Fe content ⁵⁴. Furthermore, the
185 identification of a quadruple mutant combining knockout in 3 out of the 4 genes encoding
186 NAS in Arabidopsis and a knockdown in the fourth one allowed to obtain fertile plants with
187 drastically decreased levels of NA ⁴⁸. In this quadruple *nas* mutant, Fe and NA levels are
188 lower in seeds, similar to the phenotype observed in *ysl1*. Interestingly, in NA biosynthesis
189 mutants, Fe accumulates in phloem cells indicating that NA is required to retrieve Fe from the
190 phloem and provide it to sink tissues, such as seeds ⁵⁷. This role may be shared between NA
191 and mugineic acids in graminaceous plants. On the other hand, all YSL transporters
192 characterized so far using expression in yeast or in *Xenopus* oocytes mediate the influx of
193 NA-Fe or MA-Fe complexes into cells. The finding that NA and YSL are important for
194 phloem transport of Fe does not imply that Fe is transported as NA-Fe complex in the phloem.
195 Another important player in Fe transport from vegetative organs to the seeds is the
196 oligopeptide transporter OligoPeptide Transporter 3 (OPT3). Full loss of function of *OPT3*
197 leads to embryo lethality suggesting that the substrate of OPT3 is an essential cellular
198 metabolite ⁵⁸. Even though the closest homologue of OPT3, BjGT1, is a glutathione
199 transporter ⁵⁹, transport assays in yeast failed to show OPT3 ability to transport GSH ⁶⁰.
200 Instead, expression of *OPT3* could complement the yeast *fet3fet4* mutant impaired in Fe
201 uptake, suggesting that it transports Fe complexes. The exact substrate of OPT3 remains to be
202 identified. The identification of a weak allele of *opt3*, *opt3-2* allowed investigating its
203 function at the reproductive stage ⁶¹. In *opt3-2* mutants, Fe concentration is increased in

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

219

220 **Iron loading into seed tissues**

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

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

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

269

270 **Iron storage in seeds**

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

276 Iron and other metals are not distributed evenly in seed tissues. In contrast, Fe distribution
277 follows striking patterns. For example, in *Arabidopsis* mature embryo, Fe is highly
278 concentrated around the vascular tissues^{71, 77-81}. Interestingly, the pattern of Fe distribution is
279 distinct from that of other metals: Mn is concentrated in the sub-epidermal cell layers of
280 *Arabidopsis* cotyledons in mature seeds while Zn is evenly distributed in *Arabidopsis* embryo
281^{71, 77, 78, 81}. The patterns of metal localization were initially discovered using synchrotron X-ray
282 Fluorescence imaging of intact seeds and were then confirmed and refined using additional
283 approaches. The use of Perls staining intensified by Diaminobenzidine (Perls DAB) allowed
284 identification of the precise cell type accumulating Fe as the proto endodermis of the embryo
285⁸⁰. The use of micro Particle Induced X-ray Emission (μ PIXE) allowed quantification of the
286 pattern: even though it is concentrated in proto-endodermal cells, Fe is present in other cells,
287 albeit at much lower concentrations, and the Fe concentrated around pro-vascular tissues
288 accounts for 50% of total seed Fe⁸¹. Finally, the use of Electron Dispersive X-ray imaging
289 coupled to Transmission Electron Microscopy (TEM EDX) provided higher spatial resolution
290 allowing the determination of the subcellular localization of Fe in the globoids of vacuoles of
291 proto-endodermal cells (Figure 4)^{79, 82}. This subcellular localization is in agreement with the
292 finding that mutations in *AtVIT1* (Vacuolar Iron Transporter 1) disrupt the pattern of Fe
293 distribution^{71, 79}. In *vit1* mutants, Fe is not concentrated around vascular tissues but instead
294 co-localizes with Mn in sub-epidermal cells^{71, 79}. Nevertheless, Fe is still localized in
295 vacuoles in *vit1* mutants⁷⁹. Recently, the vacuolar Mn transporter *AtMTP8* (Metal Tolerance
296 Protein 8) was shown to be responsible for concentrating Mn in sub-epidermal cells in wild
297 type seeds and Fe localization in these cells in the *vit1* mutant background^{77, 78}. In *mtp8*
298 knockout mutant seeds, Mn is concentrated around the vascular tissues together with Fe. In a
299 double mutant combining *vit1* and *mtp8* mutations, Mn and Fe are evenly distributed in
300 *Arabidopsis* embryo⁷⁸. Therefore, by creating strong sinks for Fe or Mn, vacuolar metal
301 transporters determine the pattern of metal distribution in mature *Arabidopsis* embryo. The
302 phenotype of *vit1* mutant, which survives poorly when germinated under Fe-deficient
303 conditions, indicates that adequate tissue localization in the embryo is crucial^{71, 79}. Little
304 information is available on how the Fe distribution pattern is generated during embryo

305 development. Consistent with a strong expression of VIT1 during seed development, Fe
306 patterning is already apparent at the torpedo stage at the center of the cotyledons where the
307 provascular tissue will differentiate^{71, 80}. However, in *Brassica napus*, high resolution Perls
308 DAB imaging detects Fe in the nuclei of all embryo cells at the torpedo stage⁸³. Further
309 during development, at the bent cotyledon stage, Fe is detected in cytoplasmic vesicles and
310 finally ends up in the vacuoles of the cells surrounding the vasculature in mature embryo⁸³.
311 The observation of Fe in the nucleus at the torpedo stage in *B. napus* is consistent with the
312 detection of high Fe concentrations in the nucleus of embryo cells in developing pea (*Pisum*
313 *sativum*) seeds⁸⁴. Therefore, Fe subcellular localization evolves during embryo development.
314 These changes in localization are most likely paralleled by changes in speciation and therefore
315 of Fe bioavailability. The analysis of Fe bioavailability in pea seeds at different
316 developmental stages show that Fe bioavailability in immature peas is higher than in mature
317 pea seeds⁸⁵.
318 Whether the pattern of Fe distribution in the vacuoles of cells surrounding the vasculature
319 described in Arabidopsis is conserved among angiosperm has been investigated. In beans
320 (*Phaseolus vulgaris*), Fe stores also concentrates around vascular tissues⁸⁶. Recently, this
321 observation was extended to Brassicales and even Rosids, which represent a wide group
322 including a third of the angiosperms^{87, 88}. However, in many cases the number of cell layers
323 that accumulate Fe is increased, to 2, the endodermis and one cortical cell layer in Brassicales,
324 or even more cortical cell layers in other Rosids, compared to endodermis only in
325 Arabidopsis^{83, 87, 88}. While screening representatives of the different groups of angiosperm,
326 interesting exceptions were identified in *Chenopodium quinoa* and *Carica papaya*^{87, 88}. There
327 are certainly more diverse patterns to be discovered.
328 In grains, the seeds of graminaceous species, the distribution of Fe is very different from that
329 observed in Brassicales. In grains, in contrast to Arabidopsis, the embryo represents only a
330 small volume of the seed, while the major part is constituted by the starchy endosperm. The
331 endosperm is surrounded by the so called aleurone cell layer. During germination, the
332 aleurone layer is activated by gibberellins, releases enzymes that digest the carbohydrates
333 stored in the endosperm and undergoes programmed cell death^{89, 90}. The embryo is equipped
334 with an absorptive structure called the scutellum, somewhat equivalent to human placenta,
335 which take up the nutrients from the endosperm. In grains, a large part of the Fe is
336 concentrated in the aleurone layer and another pool is found in the embryo^{69, 91, 92}(Figure 3).
337 In wheat (*Triticum durum*), the highest concentrations of Fe are found in the aleurone layer
338 and in the scutellum⁹². Separate measurements of wheat flour and bran indicated that almost

339 60% of grain Fe is in the bran which includes the aleurone layer and therefore represents the
340 major Fe pool in grains⁹³. Within the aleurone layer, Fe is concentrated together with
341 phosphorus and other minerals in globoids inside the protein storage vacuole, similar to the
342 subcellular localization observed in Arabidopsis⁷². In situ X-ray absorption spectroscopy
343 analyses have indicated that in the aleurone layer, most Fe is bound to phytate, either as Fe(II)
344 or Fe(III) in good agreement with its subcellular localization^{69, 92}. Another pool is bound to
345 citrate, which may be involved in the transport from modified aleurone cells present in the
346 crease to aleurone cells⁶⁹. In rice, Fe accumulates in the aleurone layer together with phytate
347 and in the scutellum, as observed in wheat^{91, 94}. The exact speciation of Fe in the embryo and
348 in the endosperm has not yet been determined in these tissues that contain lower Fe
349 concentration, probably because of limitations in the sensitivity of X-ray absorption
350 spectroscopy. Based on co-occurrence of elements, it has been proposed that Fe is bound to
351 phosphate (possibly as phytate) in the scutellum in wheat and to other ligands in the other
352 parts of the embryo and in the endosperm^{69, 92}. These ligands could include proteins or
353 smaller molecules such as NA, which is present in grains as in seeds of non graminaceous
354 species^{55, 95}. In wheat flour which corresponds to the starchy endosperm, NA is the main
355 ligand for Fe⁹⁶.

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

367

368 **Biofortification**

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

373 phytate has extremely high affinity for metals, which drastically limits Fe absorption in the
374 intestine ⁷⁵. Moreover, the aleurone layer is most of the time discarded during grain
375 processing, such as polishing for rice or preparation of white flour for wheat ^{93, 96, 99}. Hence,
376 biofortification strategies using targeted molecular approaches have aimed at changing both
377 the localization and the speciation of Fe in grains. Several reviews have been recently
378 published on the attempts made to biofortify wheat and rice grain⁹⁹⁻¹⁰¹. This review will
379 therefore not extensively cover the topic of biofortification and rather just highlight a few
380 examples. The initial attempt to biofortify rice grains through biotechnology used the gene
381 encoding ferritin, which provides Fe under a highly bioavailable form, driven by an
382 endosperm specific promoter ¹⁰². Although the work provided a critical proof of concept, the
383 increase in Fe content was modest and variable ¹⁰³. Probably the most efficient strategy for
384 biofortification by manipulating a single gene has been the activation or the over expression
385 of NA synthase in rice grains ⁹⁵. This resulted not only in a strong increase in grain Fe
386 concentration but also in Fe bioavailability, as NA appears to favor Fe intestinal absorption ⁹⁵.
387 More recently, expression of a Fe vacuolar transporter TaVIT2 in wheat endosperm was also
388 shown to be a relevant approach to increase Fe content and bioavailability ⁷⁰. Probably, the
389 expression of TaVIT2 in the endosperm vacuoles creates a strong Fe sink during
390 development. Subsequently, Fe becomes available when endosperm cells die and lose their
391 internal structure. Current efforts use pyramiding of several constructs to combine the benefits
392 of multiple transgenes. This way, by combining the expression of transporters that limit
393 vacuolar storage with elevated ferritin and NA synthesis, an increase in Fe concentration in
394 grains by up to 6-fold has been obtained ¹⁰⁴.

395

396 **A role for iron in the regulation of seed germination?**

397 Seed germination starts with water uptake by the dry seed and ends with radicle protrusion
398 and initiation of cell division ¹⁰⁵. This is the critical phase of plant emergence because it is
399 tightly regulated by water availability, temperature, oxygen, and light conditions. Germination
400 is also controlled by endogenous factors such as the plant hormones abscisic acid (ABA),
401 gibberellins (GA) and ethylene that play a major role in regulating early seed germination.
402 These endogenous signals regulate germination through the process of dormancy, which is an
403 endogenous block to the completion of germination of a mature seed ¹⁰⁶. Germination is
404 followed by seedling growth during which mobilization of reserves and nutrient stored within
405 the seed sustain active cell metabolism till the acquisition of autotrophy. Thus, stand

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

408 Although the role of iron in post-germinative events is quite well documented (see below) its
409 possible involvement in the regulation of germination, *i.e.* the sum of molecular events that
410 allow radicle protrusion, is rather unknown. The effect of iron on seed germination has mostly
411 been documented in the context of soil toxicity, since high Fe concentrations are toxic and
412 inhibit germination^{107, 108}, but this is a common feature to many metals. Increase in phytate-
413 degrading enzyme activity has been reported to occur during seed germination, with a
414 concomitant decline in phytate¹⁰⁹⁻¹¹¹ thus increasing iron bioavailability¹⁰⁹. Müller et al.
415 (2009) showed that hydroxyl radicals were likely to play a role in cell wall loosening during
416 radicle elongation and weakening of the endosperm of cress seeds¹¹². Although Fenton
417 reaction is a natural candidate mechanism for explaining hydroxyl radical production they did
418 not provide any evidence about its possible involvement *in vivo*. Thus, whether iron
419 availability participates to the numerous biochemical and molecular processes involved in the
420 regulation of radicle protrusion is actually not known.

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

434

435 **Iron remobilization after germination**

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

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

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

503 With its uptake into chloroplasts of the developing seedling, Fe completes its journey from the
504 senescing leaves of the mother plant to the expanding photosynthetic apparatus of its progeny,
505 via the seed.

506

507 **Conclusions and perspectives**

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

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

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

551

552 **Acknowledgements**

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

556

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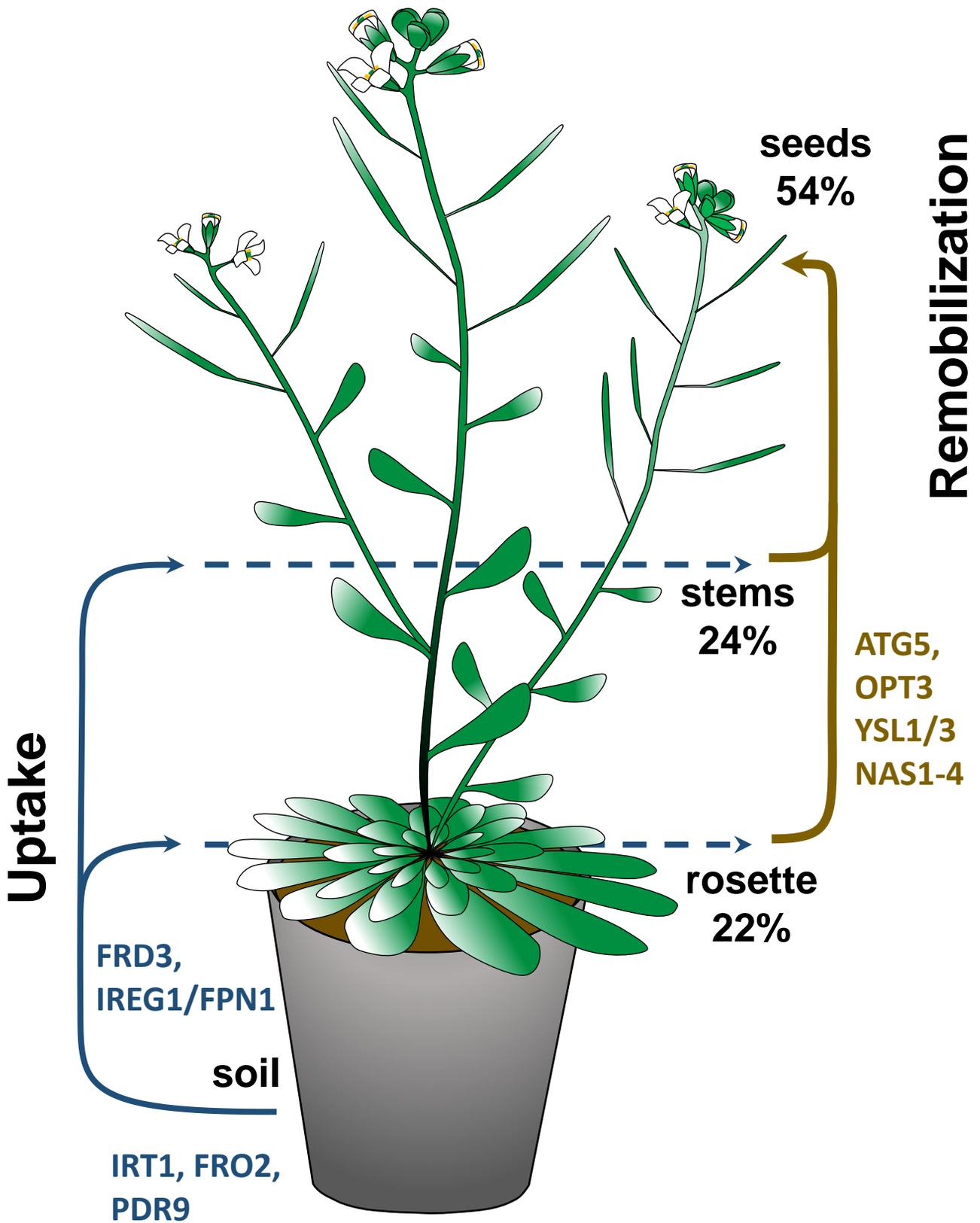


Figure 1: Fe uptake and remobilization to seeds in *Arabidopsis thaliana*. The names of the main proteins involved in uptake and translocation from roots to shoots are indicated in blue. The name of the main proteins involved in remobilization to seeds are indicated in brown. The percentages indicate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

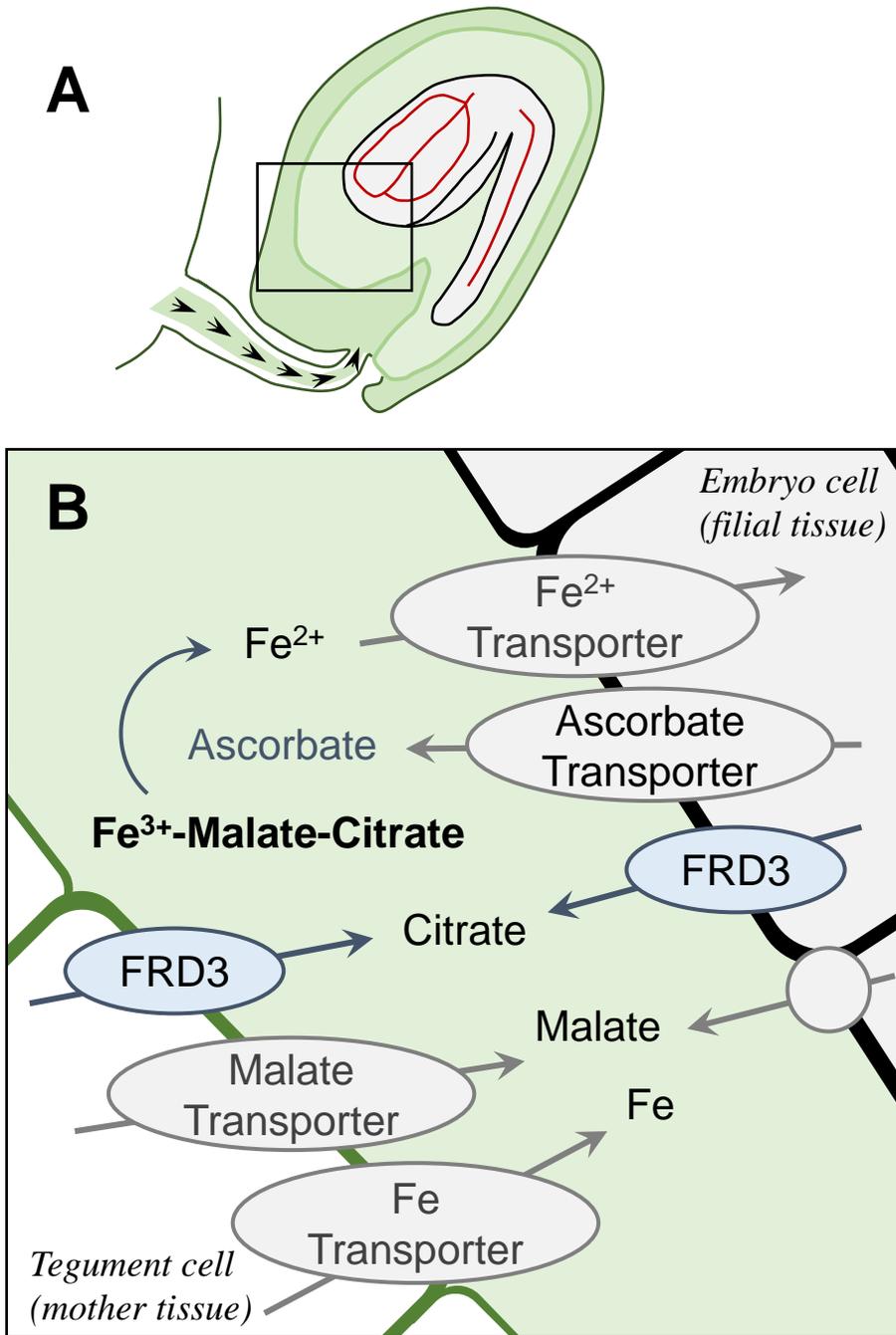


Figure 2: Fe transfer from the maternal to embryo tissues. A. Fe is transported to the maternal tissues of the developing seed but has to cross an extracellular space between the teguments and the embryo. B. Fe is bound to malate and citrate in the extracellular space separating the maternal tissues 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.

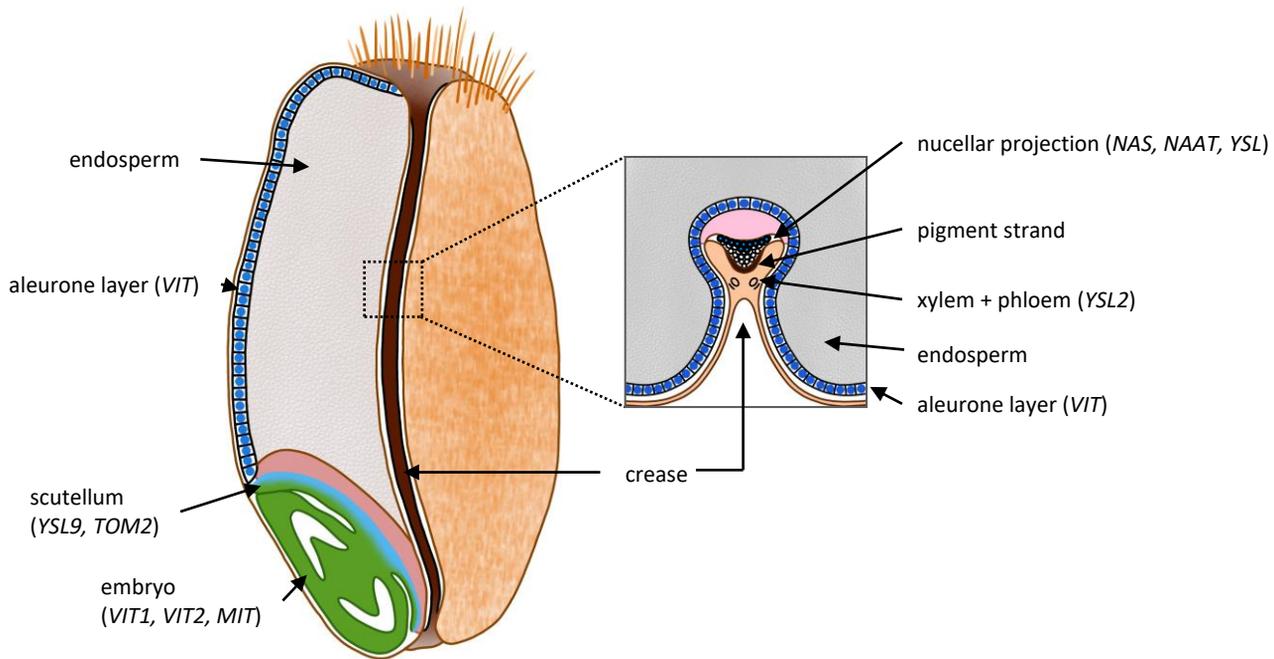


Figure 3: Fe distribution in a mature cereal grain (eg wheat grain). Fe-rich tissues and cells appear in blue, symplastic disconnections (between maternal tissues and endosperm; between endosperm and scutellum) are highlighted in pink. The size, shape and development of the crease and the nucellar projection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

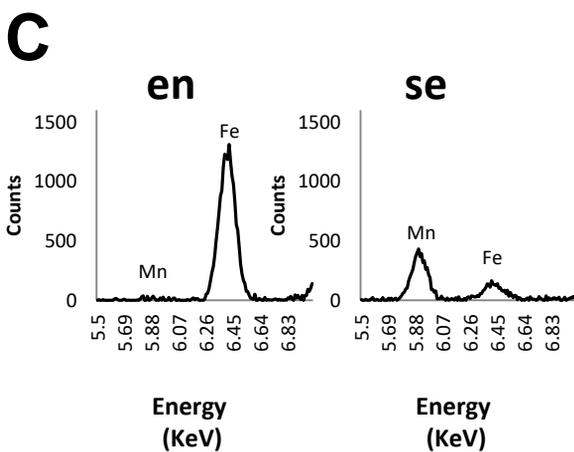
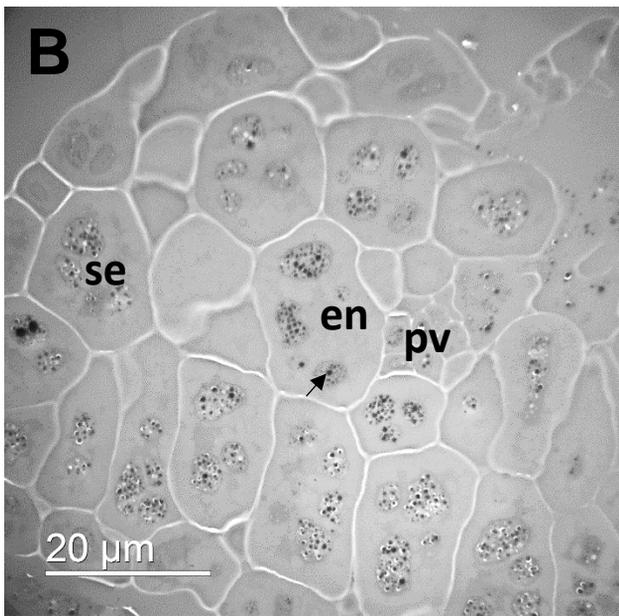


Figure 4: Fe distribution in *Arabidopsis thaliana* mature embryo. A. Perls DAB staining of Fe in the whole embryo shows high concentration of Fe (brown color) around the pro-vasculature. B. TEM micrograph of an embryo section in the cotyledon showing the pro-vasculature (pv), proto-endodermis cells (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).