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Elsbeth L. Walker
University of Massachusetts Amherst, ewalker@bio.umass.edu

Brian M. Waters
University of Nebraska-Lincoln, bwaters2@unl.edu

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The role of transition metal homeostasis in plant seed development

Elsbeth L. Walker[1] and Brian M. Waters[2]

1 Biology Department, 611 North Pleasant St., University of Massachusetts, Amherst, MA 01002
2 Department of Agronomy and Horticulture, 377K Plant Sciences Hall, University of Nebraska–Lincoln, Lincoln, NE 68583-0915

Corresponding author – Elsbeth L. Walker, email ewalker@bio.umass.edu

Abstract
For human health, transition metal accumulation in edible seeds like cereal grains is of worldwide importance because Fe and Zn deficiencies are among the most prevalent human nutritional disorders in the world. There have been many recent developments in our understanding of the patterns in which transition metals accumulate in the seeds, the identity of some specific transporters that are required for efficient seed metal accumulation, and the central role played by the ubiquitous plant metal chelator nicotianamine (NA). These and other recent discoveries will be reviewed here.

Metals are crucial for plant sexual reproduction

Mounting data indicate that metal homeostasis is crucial for normal flower development and reproduction. Although the specific roles of Fe, Zn, and Cu in floral function are not clear, plant sexual reproduction requires high metabolic activity and rapid growth. Production of gametophytes, pollen tube growth, and embryo and seed development rely on mineral micronutrients for many of the same biochemical processes that occur in other tissues. Recent transcriptomic and proteomic studies have indicated expression of Fe-requiring (cytochrome P-450, Fe-superoxide dismutase (FeSOD), and peroxidase) and Cu-requiring proteins (plastocyanin-like proteins) during female gametophyte development [1,2], and Cu-requiring (dehydroascorbate reductase (DHR)) proteins during male gametophyte development [3]. The proteome of pollen includes Fe-containing (ascorbate peroxidase (APX), thioredoxin reductase, and catalase), Cu-containing (DHR, blue copper binding protein), and Zn-containing proteins (CuZnSOD) [4,5], whereas in a transcriptomic study of germinating and growing pollen tubes there were 29 Cu-related, 32 Fe-related, and 279 Zn-related (based on KEGG annotation of the *Arabidopsis thaliana* genome) expressed genes [3]. Most of the Zn genes encode Zn-finger proteins, but CuZnSOD and carbonic anhydrase were also among them. Genes encoding iron-requiring proteins included aconitase, cytochrome P-450s, and ferritins. A surprising number of genes encoding metal transporters (COPTs, IREG3, ZIF1, ZIP12, and HMA1) were found expressed in the germinating and growing pollen tubes. Following pollen tube growth, fertilization takes place in the embryo sac. Transcriptomic profiling of this tissue implicated two Cu-binding proteins, four cytochrome P-450s, a dioxygenase (Fe-dependent), and 17 zinc finger proteins [6]. In a proteomic study of soybean seed growth [7], several Fe-requiring (lipoxygenase, succinate dehydrogenase, ferritin, APX, and peroxidase), Cu-requiring (Cu chaperone), and Zn-requiring (alcohol dehydrogenase and zinc metallopeptidase) proteins were present, while a combined microarray and proteomic approach [8] revealed a similar set of metal containing genes important for *Arabidopsis* seed growth (catalase, APX, SODs, and ferritin).

We lack fundamental information regarding delivery of metals to these important reproductive structures and their protection from metal toxicity, although recent work has provided some clues. Ferritin proteins have recently been shown to buffer free Fe and prevent oxidative damage from Fe-catalyzed formation of free radicals [9*]. Absence of ferritin proteins can result in high Fe concentrations and oxidative damage in flowers, and failure to set fruits [9*], underscoring the need for metal homeostasis during reproduction. Mutants of several transporters have resulted in infertility. *Arabidopsis* lines with mutated *yellow stripe-like* (*ysl1* and *ysl3*) genes produce few functional pollen grains, and have small seeds containing embryos arrested at various immature stages, which often fail to germinate [10]. Homozygous null mutations in *AtOPT3* (*oligopeptide transporter3*) cause early embryo arrest [11], while a partial loss-of- function allele is able to undergo embryo development, but has lower seed Fe concentration [12*]. The effects of these mutations on the plant will be discussed below. Another metal-related transporter mutant with decreased fertility is the *hma2hma4* double mutant [13]. Zinc accumulation is disrupted in this mutant, which forms sterile flowers without pollen. The phenotype could be rescued by supplying the plants with high Zn.

Pathways to the seed

Developmentally determined senescence programs have been long recognized as contributing to re-translocation of certain macronutrients and micronutrients (fig. 1a) [14–

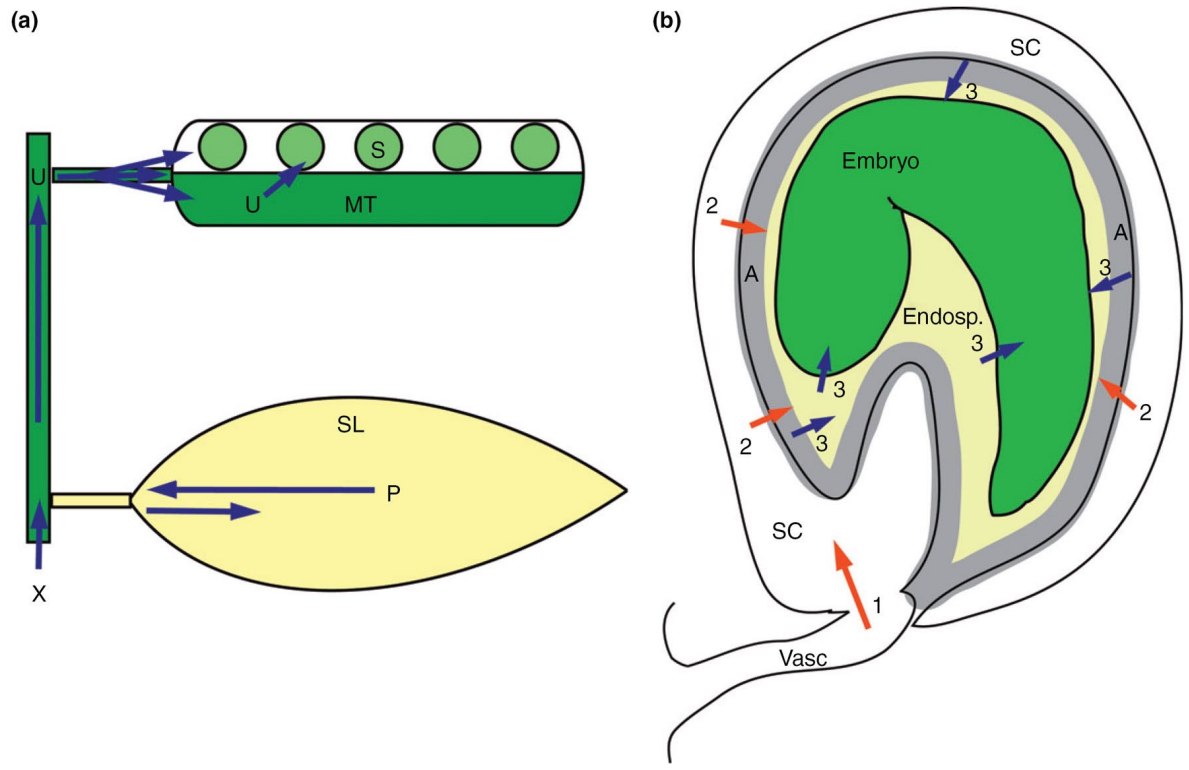

Figure 1. Movements of transition metals during seed set. (a) On the whole plant level, metals move toward apically located seeds either in the xylem stream initiating at the root (X), or in phloem sap initiating in leaves, stems or photosynthetically active tissues of developing fruits (P). Senescing leaves (SL) are net exporters of metals during seed set. The balance of import versus export can change over the course of development and in response to the metal nutrient status of the plant [23**,64]. Close to the developing seed, metals must be actively moved out of stems and taken up again (U) where they proceed into the seeds (S), or into maternal tissues (MT) such as fruit, illustrated here as a silique. Short distance translocations from these maternal tissues into seeds also occur [41,65]. (b) Generalized steps required to translocate nutrients into seeds. Cellular efflux steps are indicated by red arrows; uptake steps are indicated by blue arrows. Unloading of phloem (1) in vascular connection (Vasc) to maternal plant; efflux from maternal seed coat (SC) cells (2) into the apoplast (shaded) separating filial tissues; uptake into cells of filial tissues (3) of endosperm (pale green) and/or embryo (dark green).

16]. A relationship between leaf senescence and transition metal accumulation in grains came from the identification of the gene responsible for a major grain protein concentration (GPC) quantitative trait locus in wheat called *Gpc-B1*, and later *Nam-B1* [17–19]. Plants with functional alleles/homeologs of the gene contain higher grain Fe and Zn concentrations [20,21], and strikingly, show accelerated senescence [22]. Modern durum wheat cultivars contain a nonfunctional allele of *Nam-B1* and exhibit delayed senescence with lower GPC, Zn and Fe [19]. Whole plant mineral partitioning studies of a *NAM* RNAi knock down line supported a connection between senescence and micronutrient accumulation in the grains. Total shoot Fe, Zn, and N contents were equal, but the RNAi line failed to accumulate normal levels of Fe, Zn, and N in grain, resulting from decreased remobilization from shoots. Strikingly, remobilization normally occurs throughout the plant: from leaves, stem, peduncle, florets, and rachis, indicating that any or all of these sites load Fe and Zn to the phloem for movement to the grain. Accumulation of Fe and Zn in the stem, rachis, and glumes of *NAM* RNAi plants (fig. 2), indicates that transfer from these tissues to grain is a potential rate-limiting factor. In hydroponic experiments in which either Fe or Zn was not added to the medium after anthesis, both control and *NAM* RNAi plants remobilized a larger percentage of Fe or Zn from vegetative structures. This demonstrates that *NAM* knock down plants are capable of remobilization under some circumstances, and also shows marked flexibility in the pools of Fe and Zn that are used for reproduction [23**].

Molecules involved in metal movement to the seed

A particularly important molecule for long distance metal translocation is the non-proteinogenic amino acid, nicotianamine (NA), a strong transition metal chelator found at concentrations (20 and 500 nmol g^{-1} fresh biomass) that suggest it is a major chelator of transition metals in plants [24–27]. A tomato mutant, *chloronerva* (*chln*), that lacks NA synthase (NAS) [28–30] has marked defects in transition metal ion homeostasis, and is sterile [31,32]. Many aspects of the complex *chln* phenotype seem to reflect defects in Fe utilization, for example, chlorosis of the youngest leaves, excess Fe

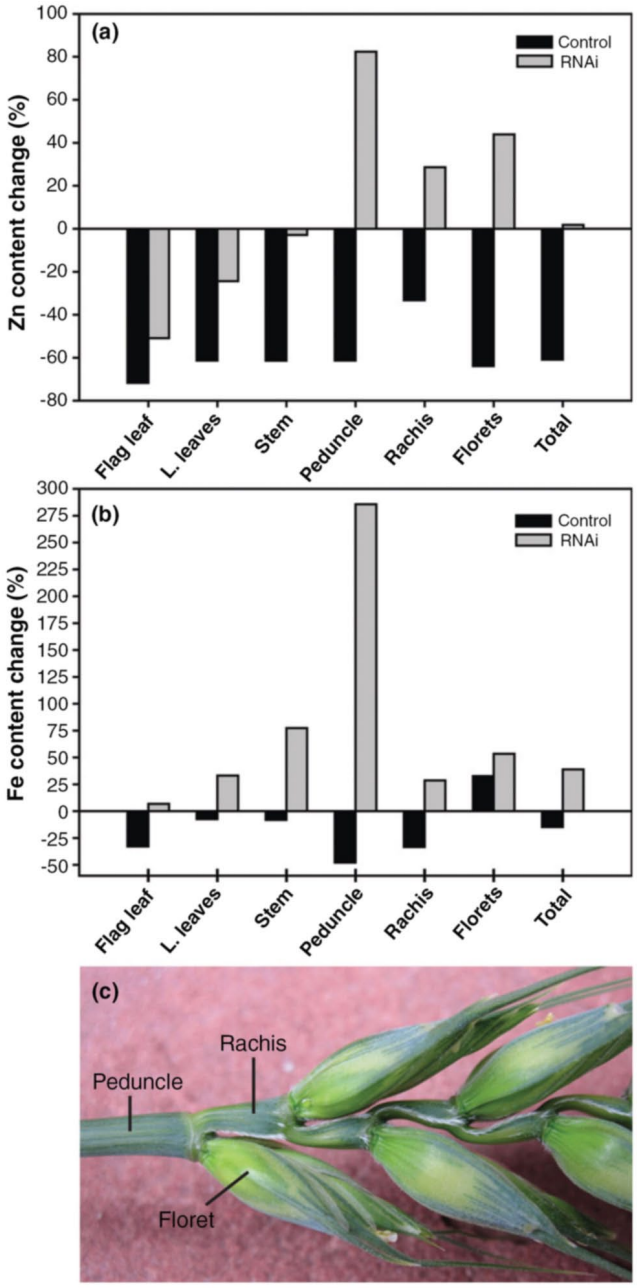

Figure 2. Zinc and Fe remobilization and accumulation in wheat during reproductive development. Shown are changes in total Zn (a) and Fe (b) contents between anthesis and grain maturity in parts of control and *NAM* RNAi line wheat plants (data from [23**]). Floret data do not include grains. L. leaves = lower leaves. (c) Photograph of wheat spike showing peduncle, rachis, and floret.

in the older leaves, and constitutive up-regulation iron uptake systems at the root [24,33]. However, Cu translocation from root to shoot is also defective in this mutant [25,34], suggesting that NA may be an important chelator of other transition metals, too.

Adding to this picture, Klatte et al. [35**] have recently reported two quadruple NAS mutants in *A. thaliana*. *nas4x-1*, which bears a partially functional *nas2-1* allele, has mild chlorosis that occurs at the time of first flowering, and is fertile, while *nas4x-2*, which bears a null allele of *nas2*, has severe chlorosis throughout life cycle and is sterile. In seeds of *nas4x-1*, Fe was significantly reduced, while Zn and Cu were normal. One model to account for this is that NA is less important for Cu and Zn homeostasis than for Fe homeostasis. Alternatively, the higher affinity of NA for Zn and Cu [27,31,36,37] favors chelation with these metals even in plants with low NA.

In rice plants with either T-DNA knockouts or antisense knock down of Os*NAS3*, Fe Zn, and Cu levels were low in grains, while Mn levels were unaffected [38**]. In two independent activation-tagged alleles of *NAS3* (*NAS3- AT*), transcript levels were increased in vegetative and reproductive structures, and the NA levels of seeds increased 9.6-fold. Seeds had increased Fe, Zn, and Cu, but no change in Mn concentration. Although polishing still removed a large fraction of the metals in the seed, *NAS3-AT* mutants had higher Fe, Zn, and Cu relative to polished WT seeds [38**]. This is an especially important consideration in the biofortification of rice, where polishing removes micronutrients associated with outer seed layers and the embryo. Overexpression of barley *HvNAS1* using a CaMV35S [39] or an actin promoter [40], similarly resulted in increased NA, DMA (a phytosiderophore synthesized from NA), Fe, and Zn in rice. Although WT and *NAS3-AT* had similar amounts of Fe bound with the anti-nutrient phytate, which blocks intestinal absorption of Fe, the activation-tagged mutant had a large increase (7×) in Fe bound in a non-phytate complex. The complex is probably a cluster of Fe with several NA ligands (Fe_xNA_x) [38**]. In feeding studies, the Fe in grains of *NAS3-AT* was more bioavailable to mice [38**].

The YSL family of transporters are related to YS1, the Fe(III)–phytosiderophore (PS) transporter implicated in primary Fe uptake in grasses, but YSL genes are found in all land plants, indicating that these transporters' substrates are not limited to Fe(III)–PS complexes. YSL proteins transport NA and/or metal–NA complexes across plasma membranes, for example, into phloem companion cells, or out of the xylem and into adjacent parenchyma cells. Several recent reports have indicated that YSLs are crucial for plant reproduction and for proper loading of metals into seeds.

In *Arabidopsis*, two *YSL* genes (*AtYSL1* and *AtYSL3*) have been implicated in moving metal-NA complexes during leaf senescence and seed production. A *ysl1* and *ysl3* double mutant displays interveinal chlorosis, and as mentioned above, decreased fertility. Both the chlorosis and fertility defects can be reversed under lower light intensity [41] and by application of Fe. The concentrations of several metals (Fe, Zn, Cu, and Mo) are altered in the double mutants, and seeds accumulate significantly less Fe, Zn, and Cu

[10,41]. During leaf senescence, both *AtYSL1* and *AtYSL3* are strongly expressed throughout the leaf, suggesting that the reproduction defects in the double mutant result from failure to mobilize Fe or other metals from the leaves to the reproductive parts of the plant. The *ysl1ysl3* plants accumulated excess Cu and Zn in rosette and cauline leaves and silique hulls [41], but failed to mobilize Zn and Cu from these tissues, although Fe mobilization appeared to occur normally [10]. When primary inflorescence stems of *ysl1ysl3* mutants were grafted onto rosettes of WT plants [42**], the grafts produced functional pollen, and seed size and germination was normal, indicating that AtYSL1 and AtYSL3 activity is required in leaves for pollen and seed production. However, metal levels in the seeds from the grafts remained low, indicating that AtYSL1 and AtYSL3 are also required for translocation within the inflorescence to achieve normal metal loading into seeds. AtYSL1 and AtYSL3 activity in phloem loading in cauline leaves or silique hulls seems to be necessary for short distance translocation of metals into the seeds [41,42**].

The *OsYSL2* gene of rice is up-regulated by Fe deficiency and is capable of transporting Fe(II)–NA and Mn(II)–NA but not Fe(III)–DMA [43]. *OsYSL2* expression in Fe replete plants is restricted to phloem companion cells of the central cylinder of roots and vascular bundles of shoots, while in Fe-limited plants expression includes essentially all vascular cells [43]. *OsYSL2* is also expressed in the vascular bundle of developing seeds, and later expressed in the peripheral endosperm and the embryo [43]. When *OsYSL2* expression was lowered using RNAi [44**], grains accumulated less Fe. When *OsYSL2* was overexpressed using an *OsSUT1* promoter, which should give expression in mature phloem of all vegetative tissues and in endosperm, large increases in Fe in seeds were observed. This higher seed Fe could result from higher *OsYSL2* expression in seeds, or from increased phloem loading in shoots, or both.

Another transporter that is crucial for loading of metals into seeds is AtOPT3. Partial loss of function alleles of AtOPT3 cause excessive Fe accumulation in vegetative structures, and low Fe accumulation in seeds [12*]. *OPT3* is included in a regulatory network of genes that are known to be involved in iron homeostasis [45]. Since OPT family members may transport glutathione [46,47] or Fe–NA [48], identification of the substrate for AtOPT3 will be important for understanding the physiological role of AtOPT3 in metal loading of seeds.

Metal localization and storage in seeds

Recent advances in imaging techniques have given new insights into metal localization in seeds. In wheat, rice, and barley, metal micronutrients are primarily localized to the embryo and outer layers of the grain, while the endosperm has very low levels [49–52]. While the embryo represents a high-density site of metal micronutrients, Fe, Zn, and Mn had largely nonoverlapping localization within the various regions of the embryo [51]. X-ray fluorescence microtomography was used to visualize Fe, Mn, and Zn localization in *Arabidopsis* seeds [53]. Similar to barley grain, these minerals were found in distinct locations within the embryo, with Fe in provascular strands, Mn on the abaxial sides of both cotyledons and Zn distributed broadly throughout the embryo. Histochemical staining of Fe in developing *Arabidopsis* seeds corroborated these results and placed Fe in endodermis, the layer of cells surrounding provascular strands [54]. The VIT1 protein transports Fe into vacuoles [53], and *vit1* mutant embryos mislocalize Fe to the abaxial sides of the cotyledons, indicating that vacuolar import of Fe is needed for proper localization within the embryo [53,54]. Remarkably, though, Fe still appears to be in protein-containing globoid structures that are most probably protein storage vacuoles in the *vit1* mutant. While it is not clear how Fe enters vacuoles in the absence of the VIT1 transporter, one possibility is that a VIT1-related protein or NRAMP family member that normally transports Mn could be responsible. This would explain why in *vit1* mutants Fe accumulates in the cells that would usually only accumulate Mn [54]. Earlier, NRAMP3 and NRAMP4 proteins were implicated in efflux of Fe from vacuoles of germinating *Arabidopsis* seeds [55], and histochemical staining of an *nramp3/4* double mutant showed that Fe remained in the provascular strands during seed germination [54]. Thus, VIT1 and NRAMP3/4 represent proteins that move Fe into and out of vacuoles during seed development and germination.

Iron, Cu, Mn, and Zn increase in growing *Arabidopsis* siliques mostly during the rapid growth phase [56], indicating that import of these nutrients keeps pace with growth. Ferritin proteins were present during early silique development, but were not present during the rapid growth phase [56], which suggests that Fe is not in excess during this time. In *Arabidopsis*, the only seed-expressed ferritin protein, FER2, accumulates in mature seeds, after siliques have accumulated maximal Fe content [56], suggesting that the role of ferritin in *Arabidopsis* seeds is not primarily Fe storage, but rather in preventing an excess of free Fe. Supporting evidence for this comes from lower FER2 protein levels in seeds of *nramp3/4* mutants (which would sequester Fe in vacuoles), and in VIT1 overexpression lines (which would sequester Fe in vacuoles) [56]. However, in pea (*Pisum sativum*) seeds the majority of total Fe was found in the ferritin form [57]. In bean (*Phaseolus*) seeds, Fe pools accessible to Perl's stain were primarily localized in cells adjacent to provascular strands [58*], similar to *Arabidopsis* seeds, while immunolocalization indicated that ferritin accumulated in distinctly separate cells. Seed ferritin protein levels decrease rapidly during seed germination [9*,59], suggesting that Fe demand is high during this phase of the life cycle. Mutants of *vit1*, *fer2*, and *nramp3/4* do not have de-

creased seed Fe concentration [9*,53,55], suggesting that storage of Fe within the embryo is not a driving force for import of Fe into seeds.

Metal translocation in reproductive tissues

The seed consists of both maternal and filial tissues. Only the maternal cells are connected to the mother plant's vascular system, while the filial tissues (aleurone, endosperm, and embryo) are separated from the maternal tissues by one or more apoplastic layers [60–62]. For micronutrients to enter the filial tissues, several transport steps will be required; at a minimum: (1) phloem unloading into seed coat tissues, (2) efflux from maternal seed coat cells into the apoplast, and (3) uptake into the filial tissues (fig. 1b). By using a stable isotope of Zn in ear culture coupled with laser ablation ICP-MS, a pathway for Zn movement to wheat grain was proposed [52]. The authors found two potential bottlenecks; transfer from the maternal phloem into the filial tissues and upstream at transfer from the stem tissue to the rachis. The genes that carry out these transport steps are unknown, but a first step to identifying them was accomplished by transcriptional profiling in several specialized cell types of developing barley grain [63]. Included among the genes expressed in these tissues are *NAS*, *YSL*, *VIT1* homologues, and members of several other families of metal transporters. Discovery of genes that carry out rate-limiting transfer steps could result in strategies to overcome these barriers to metal transport to seeds.

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