Successful reproduction requires the function of Arabidopsis YELLOW STRIPE-LIKE1 and YELLOW STRIPE-LIKE3 metal-nicotianamine transporters in both vegetative and reproductive structures.¹

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ABSTRACT
Several members of the Yellow Stripe-Like (YSL) family of proteins are transporters of metals that are bound to the metal chelator nicotianamine or the related set of mugineic acid family chelators known as phytosiderophores. Here we examine the physiological function of three closely related Arabidopsis thaliana YSL family members, AtYSL1, AtYSL2, and AtYSL3 to elucidate their role(s) in allocation of metals into various organs of Arabidopsis. We show that AtYSL3 and AtYSL1 are localized to the plasma membrane and function as Fe transporters in yeast functional complementation assays. By using inflorescence grafting, we show that AtYSL1 and AtYSL3 have dual roles in reproduction: their activity in the leaves is required for normal fertility and normal seed development, while activity in the inflorescences themselves is required for proper loading of metals into the seeds. We further demonstrate that the AtYSL1 and AtYSL2 proteins, when expressed from the AtYSL3 promoter, can only partially rescue the phenotypes of a ysl1 ysl3 double mutant, suggesting that although these three YSL transporters are closely related, and have similar patterns of expression, they have distinct activities in planta. In particular, neither AtYSL1 nor AtYSL2 is able to functionally complement the reproductive defects exhibited by ysl1 ysl3 double mutant plants.
INTRODUCTION
The transition metals Fe, Cu, and Zn are among the most important and most problematic of all the micronutrients used by plants. The importance of these metals stems from their roles as essential cofactors for cellular redox reactions involved in photosynthesis, respiration, and many other reactions. The problematic nature of these metals stems from the same distinct chemical properties that make them so valuable to living systems. These metals, particularly Cu and Fe, are highly reactive and, if over-accumulated, can cause cellular redox damage. Fe presents an additional problem for plants, because it is also only sparingly soluble in aqueous solution, and thus is typically not ‘bioavailable’ in soil (Guerinot and Yi, 1994). As a response to these key properties, plants have evolved multifaceted systems to control metal uptake by the root, translocation through the plant body, storage within tissues, and remobilization during reproduction and times of nutrient stress.

The non-proteinogenic amino acid nicotianamine (NA) is a strong complexor of various transition metals, particularly Fe(II) (Anderegg and Ripperger, 1989) and Fe(III) (von Wiren et al., 1999), as well as Cu(II), Ni(II), Co(II), Mn(II) and Zn(II) (Anderegg and Ripperger, 1989). NA is present in shoots and roots at concentrations ranging between 20 and 500 nM/g fresh weight (Stephan et al., 1990), and is present in both xylem (~20 µM (Pich and Scholz, 1996)) and phloem (~130 µM (Schmidke and Stephan, 1995)), suggesting that it is a major complexor of metals throughout the plant. Much of what we know about NA function in plants comes from studies of a mutant of tomato called chloronerva, in which the single gene encoding nicotianamine synthase (NAS) is disrupted (Herbik et al., 1999; Higuchi et al., 1999; Ling et al., 1999). The chloronerva phenotype is complex. Plants exhibit interveinal chlorosis in young leaves, and constitutively activate their root Fe uptake systems, indicating that they have inadequate Fe. However, mature leaves of chloronerva mutants contain excess Fe, implying that the Fe that is present is not being properly localized in the
absence of NA. These *chloronerva* plants also have severe defects in translocation of Cu in the xylem, indicating a clear role for NA in Cu transport. The plants are sterile, indicating that NA is important during plant reproduction. Complementing these classical studies on *chloronerva*, Takahashi et al (Takahashi et al., 2003) have developed tobacco plants that heterologously express a barley gene encoding the enzyme nicotianamine amino transferase (NAAT), which converts NA into a non-functional intermediate. Recently, the phenotype of quadruple *nas* mutants was described in *Arabidopsis thaliana* (Arabidopsis) (Klatte et al., 2009). In both studies, the plants exhibited many of the defects caused by the *chloronerva* mutation, including chlorosis and an array of reproductive abnormalities.

Several members of the well-conserved Yellow Stripe-Like (YSL) family of proteins function as metal-NA transporters (DiDonato et al., 2004; Koike et al., 2004; Roberts et al., 2004; Schaaf et al., 2004; Murata et al., 2006; Gendre et al., 2007). The founding member of the YSL family, maize Yellow Stripe1 (ZmYS1) is the primary means by which roots of grasses take up Fe from the soil. The grasses, a group that includes most of the world’s staple grains (e.g. rice, wheat and corn), use a chelation strategy for primary Fe uptake. In response to Fe starvation, grasses secrete phytosiderophores (PS): derivatives of the mugineic acid (MA) acid family that are structurally similar to NA, and that form stable Fe(III) chelates in soil (Tagaki et al., 1984). This accomplishes solubilization of the otherwise nearly insoluble soil Fe. The YS1 protein, located at the root surface, then moves the Fe(III)-PS complexes from the rhizosphere into root cells (Romheld and Marchner, 1986; Curie et al., 2001; Roberts et al., 2004).

*Arabidopsis* has eight YSL genes. Three of these (*AtYSL1, At4g24120; AtYSL2, At5g24380; and *AtYSL3, At5g53550*) are expressed strongly in the xylem parenchyma of leaves, and are down-regulated during Fe deficiency (DiDonato et al., 2004; Waters et al., 2006). We have previously shown that double mutant plants with lesions in both *AtYSL1* and *AtYSL3* display strong interveinal
chlorosis. We have hypothesized that the function of these YSL transporters in vegetative tissues is to take up Fe that arrives in leaves via the xylem (Waters et al., 2006). All of the defects displayed by ysl1ysl3 double mutants can be alleviated if excess Fe is applied to the soil, demonstrating that these growth defects are caused primarily by a lack of Fe. Intriguingly, although Fe deficiency appears to be the basis of the double mutant phenotype, the concentrations of several metals are specifically altered in the double mutants (Waters et al., 2006). AtYSL1 single mutant plants have subtle phenotypes, the most striking of which is a decrease in both NA and Fe in seeds (Le Jean et al., 2005). Interestingly, leaves of these mutants contain excess NA, while Fe levels are normal. These observations are consistent with the more obvious and extensive phenotypes exhibited by the ysl1ysl3 double mutant, and highlight the idea that AtYSL proteins affect the homeostasis of both Fe and NA.

In addition to the vegetative defects mentioned above, the ysl1ysl3 double mutant has multiple defects in reproduction. Double mutant flowers produce few functional pollen grains, and thus exhibit greatly reduced fertility. Many of the seeds that these plants do manage to produce are small and contain embryos arrested at various immature stages, which often fail to germinate. These fertility defects can be reversed by application of Fe-EDDHA solution to the soil, again demonstrating that these growth defects are caused by a lack of Fe (Waters et al., 2006). Expression of YSL1 and YSL3 is very limited in flowers and developing siliques, and furthermore, the patterns of expression of YSL1 and YSL3 are distinct and largely non-overlapping in these structures. However, expression of AtYSL1 and AtYSL3 increases markedly during leaf senescence, a period in which many minerals are remobilized from leaves, presumably for delivery into developing seeds (Himelblau and Amasino, 2001). This model is in good agreement with the accepted model for nutrient loading into seeds proposed originally by Hocking and Pate (Hocking and Pate, 1977, 1978), which suggests that metals mobilized from vegetative structures account for 20-30% of the content in seeds. Direct measurements of metals in senescing and younger
leaves demonstrated that double mutants failed to mobilize Zn and Cu from leaves. Seeds produced by the double mutant plants contained reduced levels of Zn and Cu—the same metals that failed to be mobilized out of the leaves (Waters et al., 2006). This led us to propose a model in which the activity of AtYSL1 and AtYSL3 in leaves was required for correct localization of metals into the seeds. However, seeds also had low Fe levels, even though Fe appeared to be mobilized normally from leaves of the double mutants.

Here we further investigate the role(s) of AtYSL1 and AtYSL3 in allocation of metals into various organs of Arabidopsis. AtYSL1 and AtYSL3 are localized to the plasma membrane, and each is capable of suppressing the growth defect of yeast lacking normal Fe uptake, indicating that the most likely biochemical function for these proteins is in uptake of Fe(II)-NA complexes. We have used inflorescence grafting to determine the relative roles of AtYSL1 and AtYSL3 in leaves and inflorescences during seed development. These proteins are found to have dual roles: activity in the leaves is required for normal inflorescence development, while activity in the inflorescences themselves is required for proper loading of metals into the seeds. We have further examined the effect of over-expressing AtYSL3, which resulted in a small increase in Cu in shoots, and have demonstrated that the AtYSL1 protein, when expressed from the AtYSL3 promoter, can only partially rescue the phenotypes of the ysl1ysl3 double mutant, indicating that these proteins have distinct biochemical activities. A third AtYSL from the same subgroup of the YSL family, AtYSL2, also only partially complements the phenotypes of ysl1ysl3 double mutants, suggesting that although these three YSL transporters are closely related, they have distinct activities in planta.

RESULTS
Suppression of Fe growth defects in yeast using AtYSL1 and AtYSL3
An unusual phenomenon was observed while cloning AtYSL3 cDNA for use in yeast functional complementation tests. We made at least seven independent
attempts to clone AtYSL3 cDNA, both by screening cDNA libraries and by RT-PCR. Clones were obtained in each attempt, but each time the cDNAs encoded only partial (in the case of clones derived from cDNA libraries—one attempt) or mutated versions of the cDNA (in the case of clones derived from RT-PCR—6 independent attempts). Mutations were either induced through apparent polymerase errors during PCR that caused frame shifts (two independent cloning attempts), or by cloning of cDNA with one or two retained introns (four independent cloning attempts). These products are present in very low levels in our RT-PCR reactions—the vast majority of RT-PCR product is the correct size, and direct sequencing of PCR products confirms the presence of a large excess of fully spliced, expected cDNA product. We conclude that even low levels of expression of AtYSL3 in E. coli are causing lethality, and thus the only clones we obtained in E. coli were ones that do not encode AtYSL3 protein. We have made use of strains of E. coli that maintain plasmids at very low copy number (Copycutter™, Epicenter), hoping that this would help to keep expression low and allow growth. However, this approach, too, was unsuccessful: only incorrect sized clones with restriction patterns indicative of retained introns were obtained, and these were not further characterized by sequencing. Instead, we used direct cloning of AtYSL3 in yeast through homologous recombination (Oldenburg et al., 1997), followed by sequencing to confirm that correctly spliced, and non-mutated clones were obtained. This strategy completely circumvents the need for successful propagation of the AtYSL3 cDNA in E. coli. The sequence of the cDNA clone we obtained in yeast has one change relative to the canonical At3g53550 sequence, a substitution replacing a positively charged Lys with another positively charged amino acid, Arg in a weakly conserved region of the protein (Supplemental Figure 1). Cloning of YSL1 cDNA was described previously (Waters et al., 2006).

We tested whether AtYSL1 and AtYSL3 are capable of transporting Fe-NA and Fe-PS complexes using a yeast phenotypic suppression (functional complementation) assay (Figure 1). At least three independent replicates were
used to confirm each assay. In this assay, *fet3fet4* yeast were transformed with the *YSL*-expressing plasmids, and with the empty *pYES6/CT* vector, which serves as a negative control. Two positive controls, *ZmYS1* and/or *OsYSL15*, were also included. *OsYSL15* is the rice ortholog of *ZmYS1*, and like *ZmYS1*, *OsYSL15* transports both Fe(II)-NA and Fe(III)-PS complexes (Inoue et al., 2009; Lee et al., 2009). To allow us to control the level of *YSL* expression in yeast, we made use of the beta-estradiol regulated expression system developed by Gao and Pinkham (Gao and Pinkham, 2000). This system is used to achieve dose-dependent gene expression levels and further confers a tightly regulated off state, so that strains can be grown without expression of the target proteins (negative control). To demonstrate viability of the strains, all were grown under permissive conditions (50 µM Fe citrate; Figure 1A, B and C). When Fe(II) was provided in un-chelated form (as 3 µM FeSO₄; Figure 1A), both *AtYSL1* and *AtYSL3* failed to restore growth, but when NA was provided along with Fe(II) (3 µM FeSO₄ with 8 µM NA; Figure 1A), suppression of the *fet3fet4* growth defect was observed for both strains. To demonstrate that growth is dependent on *YSL* expression, beta-estradiol was withheld from the medium (Figure 1A), and growth ceased. This result indicates that these genes encode transporters of Fe(II)-NA complexes.

To assay whether *AtYSL1* and *AtYSL3* transporters can use Fe(III)-PS as a substrate for transport, the strains were grown on 10 µM FeCl₃, and on 10 µM FeCl₃ plus 10 µM 2'-deoxymugineic acid (DMA; a phytosiderophore; Figure 1B), both in the presence of the inducer beta-estradiol. Because Arabidopsis neither makes nor uses PS, neither *AtYSL* was expected to allow growth on Fe(III)-PS medium. Surprisingly, *AtYSL3* was able to complement growth in the presence of DMA, but not on FeCl₃ alone, suggesting that this protein is able to transport Fe-PS complexes. As expected, *AtYSL1* failed to complement on medium containing Fe(III)-PS. To demonstrate that growth is dependent on *AtYSL3* expression, beta-estradiol was withheld from the medium (Figure 1B). Suppression of the *fet3fet4* growth defect on Fe(III)-PS still occurred when the
strong Fe(II) chelator 4,7-biphenyl-1,10-phenanthroline-disulfonic acid (BPDS) was used to remove any residual Fe(II) from the Fe(III)-DMA medium (Figure 1B), indicating that AtYSL3 transports Fe(III)-DMA. The ability of AtYSL3 to use Fe(III)-PS as a substrate could indicate that this transporter’s in vivo substrate is Fe(III)-NA. To test this, we grew strains on medium that contained 10 µM FeCl₃ plus 15 µM NA (Figure 1C), both in the presence of the inducer beta-estradiol. Neither AtYSL1 (not shown) nor AtYSL3 allowed growth on medium containing Fe(III)Cl₃ and NA.

Localization of iron in ysl1ylsl3 double mutant seeds
We have previously shown that the seeds produced by ysl1ylsl3 double mutants are low in Fe, Zn, and Cu (Waters et al., 2006). We used synchrotron x-ray fluorescence microtomography to visualize metals directly in seeds. In Arabidopsis seeds, Fe is most strongly localized to the provascular strands of the hypocotyl, radicle and cotyledons (Kim et al., 2006). In seeds of ysl1 and ysl3 single mutants, localization of iron (Figure 2B and C) is similar to WT. The amount of iron in ysl1 mutant seeds appears to be somewhat lower than WT, consistent with previous analysis (Le Jean et al., 2005). The ysl1ylsl3 double mutant seeds have much reduced Fe (Figure 2D; note scale). Double mutant seeds are small, and frequently deformed (Waters et al., 2006), and the deformation of the seed shown is apparent (Figure 2D). However, the pattern of iron localization to the provascular strands of the cotyledons and radicle is retained in the double mutant when viewed without scaling relative to the other samples to allow clear visualization of the low levels of iron that are present in the embryo (Figure 2E). Owing to the deformation of the seeds of the double mutant, only five of the six provascular strands of the cotyledons are visible (three at top right, two at bottom right; Figure 2E), and the provascular strand of the radicle is also present. Notice that a low iron signal is present around the entire embryo (Figure 2A-D), and this is similar in amount in all four samples. Thus, the loss of AtYSL1 and AtYSL3 activity has no impact on the localization of Fe within Arabidopsis seeds, although the amount of Fe is markedly reduced.
Elemental values obtained via ICP-MS and SXRF differ considerably, and comparison is not technically accurate. Volume-averaged methods average the values over the entire volume of the seed and each value is the average of thousands of seeds. Spatially resolved metal analysis via a microprobe collects the abundance of one 10 µm thick section of one seed, and depending on the heterogeneity of the element (which for Fe, is high) the values differ widely. The localization of Mn, Cu and Zn (Supplemental Figure 2) in ysl1 and ysl3 single mutants, as well as ysl1ysl3 double mutants was similar to WT, again indicating that deficiency for AtYSL1 or AtYSL3 does not disrupt the localization of these metals within the seeds, even though the levels of these metals are altered in the double mutants.

Localization of AtYSL1 and AtYSL3 proteins in planta
Both AtYSL1 and AtYSL3 are predicted to be plasma membrane proteins, and their activity in yeast (Figure 1) is consistent with this localization, since yeast growth should rely on Fe transport across the plasma membrane. To determine the localization of AtYSL1 and AtYSL3 more conclusively, we made fusions of each gene to GFP. For AtYSL1, we placed a AtYSL1 cDNA clone under the 35S promoter, and fused GFP at the carboxyl terminus of the protein (35S-YSL1-GFP). This construct was introduced into onion skin cells using microprojectile bombardment (Figure 3A-C). The pattern of fluorescence observed for the AtYSL1-GFP fusion protein is consistent with localization to the plasma membrane: the signal is at the periphery of the cell, and does not deviate around the nucleus (Figure 1A, B and C). As a positive control for cytosolic localization, we bombarded with a construct containing soluble GFP under a 35S promoter was also introduced (35S-smGFP; Figure 3D-F). Fluorescence was observed in the cytosol and nucleus, as expected. For AtYSL3 localization, the whole gene, including its native promoter, was fused to GFP, again, at the C-terminus (YSL3-GFP). As a control for tonoplast localization, a construct containing the gene for the tonoplast localized protein TIP1 was also examined. Arabidopsis plants were stably transformed with these constructs, and the fluorescence patterns were
observed in roots of the transformed plants. The signal from YSL3-GFP is again located at the periphery of the cells, and does not deviate around nuclei (Figure 3G, H and I). By contrast, the TIP1 signal deviated around nuclei as expected for a tonoplast protein (Figure 3J, K, L; see arrows). For AtYSL3, green fluorescence was strongest in elongated cells within the vasculature of both leaves (not shown) and roots (Figure 3G, H and I), apparently xylem parenchyma. Strikingly, fluorescence for AtYSL3 was only rarely observed at the apical or basal ends of cells, but was instead found almost exclusively on the lateral plasma membranes. This pattern is very similar to that of AtYSL2 (DiDonato et al., 2004) and suggests similar functions for these two closely related proteins. This pattern is consistent with a role for these proteins in the lateral movement of metals within veins.

Grafting of \textit{ysl1ysl3} inflorescences
As noted above, reproduction of \textit{ysl1ysl3} double mutants is impaired (Waters et al., 2006). Pollen function is severely reduced, and seed development is incomplete in most cases. In addition, seeds produced by \textit{ysl1ysl3} double mutant plants contained reduced levels of Zn and Cu—the same metals that failed to be mobilized out of the leaves. The double mutant seeds also contained reduced levels of Fe. We hypothesized that the reduced levels of metals in the seeds produced by \textit{ysl1ysl3} double mutants results from decreased metal translocation into reproductive structures from senescing leaves (Waters et al., 2006). To investigate further, we grafted young (~3-7 cm) primary inflorescence stems of \textit{ysl1ysl3} double mutant plants onto WT plants, and then allowed flowering and seed set to proceed. If the metal levels of the seeds are dependent on the function of AtYSL1 and AtYSL3 in the vegetative parts of the plant, grafting should rescue the reproductive phenotypes of the double mutant. As a control, we also made self-grafts of WT plants. We attempted to perform self-grafts of the \textit{ysl1ysl3} double mutants, and grafts of WT inflorescences onto \textit{ysl1ysl3} rosettes, but were not able to obtain viable grafts in spite of repeated attempts. We therefore present comparative data obtained from un-grafted
double mutant plants. Visual inspection of the grafted ysl1ysl3 flowers indicated that anther stunting was alleviated and pollen was released from anthers of the grafted flowers. The measures of seed set (a quantitative proxy for pollen function, since non-functional pollen results in failure of seed set; Figure 4A) and seed weight (Figure 4B) indicate that AtYSL1 and AtYSL3 function is required in leaves for normal pollen and seed development. Both seed set and seed weight from the grafted plants were significantly higher than un-grafted double mutant plants, and were furthermore not significantly different from WT self-grafts (by Student’s T-test). These results indicate that AtYSL1 and AtYSL3 functions are required in the rosettes (stems/leaves/roots) to accomplish normal pollen and seed development.

We also tested whether the ysl1ysl3 seeds produced by grafted inflorescences would germinate normally. Seeds from un-grafted double mutant plants show severe germination defects on soil ((Waters et al., 2006); Figure 4C), with only 31% of seeds germinating. By contrast, germination of seeds produced from grafted inflorescences (89%) was not significantly different from seeds of WT self-grafts (91%).

We measured the metal content of the seeds produced by ungrafted WT plants, WT self-grafts, ysl1ysl3 grafts to WT, and ungrafted ysl1ysl3 plants. By contrast to the rescue of pollen function, seed development, and germination caused by grafting, the content of Fe, Zn, and Cu in the seeds from grafted plants remained low (Figure 5). For each of these metals, there was a trend towards the normal metal level in the ysl1ysl3 seeds that developed from the grafted inflorescences. However, the metal levels of the seeds produced from grafted ysl1ysl3 stems are not significantly different (by Student’s T-test) from the metal levels in seeds of the un-grafted double mutant plants. Seeds from both grafted and un-grafted ysl1ysl3 inflorescences are significantly lower than un-grafted WT and WT self grafts with respect to Cu and Zn. The Fe levels of seeds produced by ungrafted ysl1ysl3 plants were not significantly lower than WT levels, but the Fe levels of seeds from the grafted ysl1ysl3 inflorescences was significantly lower than that of WT or WT self-grafts. These results indicate that AtYSL1 and AtYSL3 function
are required in the grafted portion of the plants, *i.e.*, in the inflorescence stems or cauline leaves, the flowers themselves, or the developing fruits and seeds, in order for the seeds to contain normal metal levels at maturity. By comparing ungrafted WT plants to WT self-grafts, we were able to observe that grafting caused a large decrease the Mn content of the seeds produced by grafted branches, but the other metals measured in this study were not affected by grafting *per se*.

**Phloem sucrose concentrations in ysl1yls3 double mutants**

The normal development of pollen and seeds in ysl1yls3 grafts caused us to question whether the recovery of pollen and seed development is caused by YSL-mediated movement of metals, or to an indirect affect on bulk flow in phloem. Since ysl1yls3 double mutants are chlorotic, they presumably have lower than normal rates of photosynthesis, and thus may export less sugar via phloem. Lower sugar transport could have two distinct effects. One is that, to drive phloem transport of Fe to the seeds, adequate sucrose in the phloem is required. In this model, pollen and seed defects could be the result of reduced metal translocation that is a secondary effect from generally defective phloem transport. Alternatively, sugar itself must be needed for pollen and seed development, thus the simple lack of carbohydrate experienced by the chlorotic ysl1yls3 plants could be the cause of the pollen and seed defects.

Previously published experiments in which ysl1yls3 double mutants were treated with ferric ammonium citrate (Waters et al., 2006) can be used to address the concern that pollen and seed failure result from a lack of carbohydrate. Ferric ammonium citrate treatment corrected the chlorosis of ysl1yls3 double mutants, suggesting restored photosynthesis in treated double mutant leaves. However, ferric ammonium citrate treatment only weakly affected plant fertility. In this experiment, normal photosynthesis in leaves but loss of function of *AtYSL1* and *AtYSL3* results in poor recovery of pollen and seed development, strongly suggesting that it is metal transport, not sugar production that causes the pollen
and seed weight defects of the double mutant. To support this idea, we measured the level of sucrose in the phloem of WT and *ysl1ysl3* plants (Figure 6). The glucose content in the phloem exudates was below the detection limit (50 nmole/g fresh weight; not shown), suggesting that the contamination caused by cell damage in the exudates is low. The amount of sucrose in the phloem of the double mutants did not differ from that in WT plants, suggesting that sucrose translocation and bulk flow in the phloem of the double mutants is normal.

**Over-expression of *AtYSL3* in planta**

Over-expression is an important tool that can be used to determine the function of a gene. To accomplish over-expression, the gene is typically placed under the control of a strong constitutive promoter to cause high expression levels in most or all cells. This approach did not work well for *AtYSL* genes, as this type of over-expression gave rise to seedlings that did not thrive, and did not produce consistent phenotypes (not shown). As an alternative, we added multiple copies of the 35S enhancer to the native *AtYSL3* promoter, which then drives expression of the downstream gene. This arrangement is expected to lead to an enhancement of expression but in the normal location(s), without causing ectopic expression (Weigel et al., 2000; Mora-Garcia et al., 2004; Tian et al., 2004). Quantitative RT-PCR experiments demonstrated that only one of three over-expression lines tested had increased mRNA levels in leaves (Figure 7A). Expression in cauline leaves, and in the flowers and flower buds was close to normal (Figure 7B, C and D). To determine whether over-expression of *AtYSL3* in vegetative tissues caused changes in metal accumulation, we examined the levels of metals in vegetative leaves (Figure 7E) and in seeds (Figure 7F). In all three lines, the Cu levels of the leaves were significantly higher than normal. Fe and Zn levels were not affected in the over-expression lines. In addition, the leaf Mn level of only a single line (7.1) was affected. In the seeds, no significant changes in the level of any metal were observed, with one exception: one line produced seeds with a small but significant decrease in Zn. However, since the Mn and Zn changes were only associated with a single line, and moreover did
not correlate with expression levels, their importance is not clear. The lack of clear changes in metal content in seeds may reflect the weak or non-existent over-expression indicated by RT-PCR results from flowers, buds, and especially cauline leaves.

In an effort to uncover subtle phenotypes associated with over-expression of *AtYSL3*, and because over-expression of *AtYSL3* was strongest in vegetative tissues, we examined whether the lines were resistant or sensitive to Fe deficiency stress. In this experiment, seeds were germinated on MS medium that lacked Fe, and the total chlorophyll level of the seedlings, which reflects the level of Fe deficiency chlorosis, was measured at various time points. Line 5.8, which had the highest expression level for *AtYSL3* mRNA (Figure 7A), was mildly resistant to prolonged Fe deficiency, with significantly higher chlorophyll levels at later time points, *i.e.*, 18-24 days of Fe starvation (Figure 7G). The two lines with weaker expression showed no significant differences from wild type Col0 plants.

**Complementation of ysl1ysl3 double mutants by other YSL genes**
The YSL family contains three well-conserved clades that are present in all plants (Curie et al., 2009). A key question concerning the YSL family is whether each member has a distinct biochemical function, or instead whether closely related members of the family share overlapping biochemical functions, and differ only in their expression patterns. *AtYSL1* and *AtYSL3* both belong to the same clade, and so might have similar or identical biochemical functions. This model explains the mild phenotype of the single *ysl1* mutant (Le Jean et al., 2005) compared to the severe phenotype of *ysl1ysl3*: the two genes could have overlapping expression patterns but encode functionally equivalent proteins. This does not completely explain the ysl1ysl3 phenotype, however, because in flowers, fruits and seeds there is little or no overlapping expression between *AtYSL1* and *ATYSL3*, and thus straightforward functional redundancy may not explain these attributes of the double mutants.
To test this, we used a complementation approach in which a cDNA encoding *AtYSL1* was placed under the control of the *AtYSL3* promoter, and the resulting construct was used to transform *ysl1ysl3* double mutant plants. If *AtYSL1* and *AtYSL3* have highly similar (or identical) biochemical functions, then the phenotypes associated with the double mutant (chlorosis, infertility, poor seed viability) should be alleviated or reversed. Complementation using *YSL3p:YSL1* provided only partial rescue of the phenotypes associated with the *ysl1ysl3* mutant. Chlorophyll levels in four independent lines were intermediate between those of WT and the un-complemented double mutant (Figure 8A and B), and were significantly (by Student’s T-test) different from both. Thus, *AtYSL1* can only partially complement *AtYSL3* transport function *in planta*. The reciprocal experiment (*AtYSL1* promoter driving *AtYSL3* expression) was not performed, because *AtYSL3* cDNA cannot be maintained in *E. coli*, and thus cannot be readily manipulated to generate the appropriate construct. However, a positive control, a *AtYSL3* genomic clone that was fused to GFP for localization studies, and which contains the same promoter as used in this experiment, has been used to transform the double mutant, and complements the chlorotic double mutant phenotype completely (Figure 8C).

Complementation of reproductive phenotypes using *YSL3p:YSL1* was even less successful (Figure 8D and E). The construct used was not able to correct seed development (seed weight; Figure 8D), since the seeds produced by plants containing *YSL3p:YSL1* were the same weight as double mutant seeds. In addition, the fertility defect of the plants was not corrected by the *YSL3p:YSL1* transgene; plants carrying the construct produced a similar number of seeds as the double mutant plants. Both seed amount and seed weight are normal in *ysl1ysl3* plants complemented with *YSL3::GFP* (not shown). Thus, *AtYSL1* and *AtYSL3* appear to have related but distinct functions *in planta*.

A third Arabidopsis YSL family member, *AtYSL2*, is present in the clade that
contains \textit{AtYSL1} and \textit{AtYSL3}. The pattern of expression of \textit{AtYSL2} is similar to that of \textit{AtYSL1} and \textit{AtYSL3} in that expression is strongest in leaf veins, and is negatively regulated by Fe deficiency. Also like \textit{AtYSL1} and \textit{AtYSL3}, \textit{AtYSL2} is a plasma membrane protein, and is capable of transporting Fe(II)-NA (DiDonato et al., 2004). Thus, we tested whether \textit{AtYSL2} could substitute for \textit{AtYSL3} in the double mutant, by constructing a \textit{YSL3p:YSL2} gene in which \textit{AtYSL2} cDNA is driven by the \textit{AtYSL3} promoter. In three independent lines carrying this construct (in a \textit{ysl1ysl3} double mutant background), chlorophyll levels were intermediate between WT Col0 and \textit{ysl1ysl3} mutants, indicating that \textit{AtYSL2} only partially complements \textit{AtYSL3} function (Figure 8A and B). Both seed weight and seed production were unimproved in \textit{ysl1ysl3} plants carrying the \textit{YSL3p:YSL2} construct (Figure 8D and E). Thus, \textit{AtYSL2} and \textit{AtYSL3} also appear to have related but distinct functions \textit{in planta}.

**DISCUSSION**

Reproduction requires \textit{YSL1} and \textit{YSL3} in distinct locations

An accepted model for nutrient loading into seeds (Hocking and Pate, 1977, 1978) suggests that metals mobilized from vegetative structures account for 20-30% of the metal content of seeds. Thus, starting from the simple hypothesis that \textit{YSL1} and \textit{YSL3} activity in leaves allows metal movement from senescing leaves into seeds, we performed grafting experiments that separated gene expression in the reproductive structures (inflorescence stems containing cauline leaves, flowers, fruits and seed) from gene expression in the vegetative structures (rosettes and roots). By doing this we uncovered a more complex pattern than was initially anticipated. Expression of \textit{YSL1} and \textit{YSL3} in the rosettes and roots is necessary for successful formation of pollen and for completion of seed development (Figure 4), suggesting that metals that are translocated from senescing leaves are used for the manufacture of reproductive structures. However, \textit{YSL1} and \textit{YSL3} are also required more locally—within the inflorescences—in order for loading of metals into the seeds to proceed normally (Figure 5). These data indicate that short range translocation within the
inflorescence itself is more important than long range translocation from shoot or root to inflorescence for metal deposition into seeds. In previous work (Waters, 2006) we showed that YSL1 and YSL3 are poorly expressed in flowers, fruits and seeds, and moreover that their expression does not overlap in these structures, suggesting that YSL1 and YSL3 activity within these structures is unlikely to be responsible for loading of metals to seeds. Instead, we propose that it is activity in the cauline leaves or the inflorescence stems that is required for the short distance translocation of metals into the seeds themselves. According to publicly available microarray data and our own unpublished results, both YSL1 and YSL3 are strongly expressed in cauline leaves and moderately expressed in inflorescence stems, making these structures excellent candidates for key sites of metal export for re-translocation into developing seeds in Arabidopsis.

This model is consistent with the results of classic experiments performed by Grusak, who demonstrated that Fe applied to the pod wall of peas was rapidly re-translocated into developing seeds (Grusak, 1994), and with more recent experiments examining whole-plant dynamics of mineral flow (Waters and Grusak, 2008). In addition, our finding that the seeds of the grafted plants have normal germination in spite of their lower Fe, Zn and Cu levels, indicates that the germination defects of the double mutant are likely due to seed developmental arrest, not to metal content per se.

Formally, failure of pollen and seed development in the double mutant could result from aberrant metal homeostasis, or, alternatively could arise from diminished sugar translocation resulting from reduced photosynthetic capacity of the chlorotic double mutants. Lower sugar transport could have two distinct effects. One is that, to drive phloem transport of Fe to the seeds, adequate sucrose in the phloem is required. In this model, pollen and seed defects could be the result of reduced metal translocation that is a secondary effect from generally defective phloem transport. Alternatively, sugar itself must be needed for pollen and seed development, thus a simple lack of carbohydrate in chlorotic
ysl1ysl3 plants could be the cause of the pollen and seed defects. We found that the levels of sucrose in the phloem of the double mutant plants was similar to WT levels, suggesting that low carbohydrate is not causing reproductive phenotypes. Moreover, ferric ammonium citrate treatment corrected the chlorosis of ysl1ysl3 double mutants, suggesting restored photosynthesis, but only weakly affected plant fertility, again suggesting that it is metal transport, not sugar production that causes the pollen and seed weight defects of the double mutant (Waters et al., 2006). We conclude that pollen and seed development in the double mutant fails because metal transport from senescing leaves is defective.

The transporter activity of AtYSL1 and AtYSL3 in yeast
Here we have shown that AtYSL1 and AtYSL3 suppress the Fe specific growth defect of the yeast fet3fet4 mutant, indicating that these YSLs are Fe transporters. In previous work, we used a non-inducible expression system for functional testing of YSL proteins using yeast growth complementation (DiDonato et al., 2004). However, the vector used, pFL61, caused unanticipated growth changes in the alternative yeast strain (ftr1) used in similar experiments conducted by other groups (Schaaf et al., 2005). In this work, by carefully controlling YSL expression from a BE-inducible promoter, we have demonstrated that growth is dependent on both expression of each YSL gene, and on the presence of the appropriate chelator (NA or PS). In medium with an equal amount of Fe, but no chelator, growth limitation occurs. In medium with an equal amount of chelator and iron, but without BE, growth limitation occurs. This strongly implies that Fe-NA or Fe-PS complexes are required by these YSLs as substrates for transport. Although Fe(II)-NA transporter activity was expected for AtYSL1 and AtYSL3, it was surprising to find that Fe(III)-PS is apparently a useable substrate for transport by AtYSL3, since PS, including DMA used in the assay, are not produced by Arabidopsis. Only grass species (e.g., rice, maize, wheat, etc) produce PS, and so only these species would be expected to have YSLs that transport PS. One explanation for this activity of AtYSL3 is that this protein might possess the ability to transport Fe(III) bound to NA. Fe(III)-NA
complexes are expected to occur in all plants (von Wirén et al., 1999), including Arabidopsis, and DMA, being structurally very similar to NA, could be substituting for NA to form a transportable complex. When medium containing both Fe(III) and NA were used in the growth assay, no functional complementation was observed. This negative result leaves the question of Fe(III)-NA transport via AtYSL3 open, since the failure of complementation could reflect a true lack of this transport activity for AtYSL3, or could reflect a problem with the assay conditions, e.g., if Fe(III)-NA complexes do not form, or are unstable under the conditions used in the complex medium needed for yeast growth. Since AtYSL3 appears to possess a distinct biochemical function that cannot be completely replicated by AtYSL1 or AtYSL2 (see below), it is tempting to speculate that the latter explanation may be correct.

The ysl1 ysl3 double mutants exhibit marked alterations in Zn and Cu homeostasis, especially in the seeds, but also in leaves during senescence (Waters et al., 2006). This could come about because of a direct effect in which AtYSL1 and AtYSL3 are transporters of these metals, or could be an indirect effect in which altered Fe homeostasis causes secondary effects on the levels of other metals. We have also examined whether AtYSL1 and AtYSL3 are capable of transporting Zn-NA complexes and Cu-NA complexes using yeast functional complementation with strains defective in copper or zinc uptake, but we have not observed any evidence of Cu or Zn uptake (not shown), although the AtYSL2 protein does appear to transport Cu-NA in these assays (DiDonato et al., 2004). We are reluctant to ascribe any functional significance to these negative results. Additional assays systems must be developed before a conclusion can be reached regarding the capacity of these proteins to move substrates other than Fe(II).

Functional equivalency of YSL transporters in planta
We have also examined the functional equivalency of the three proteins in the YSL1/YSL2/YSL3 clade of Arabidopsis, and find that these proteins, in spite of
their strong, full length sequence similarity (AtYSL1 and AtYSL2 are 79% similar; AtYSL2 and AtYSL3 are 86% similar), do not completely functionally complement each other’s activities in planta. Partial complementation of AtYSL3 activity was observed in vegetative structures, where expression of AtYSL1 and AtYSL2 from the AtYSL3 promoter produced plants that were less chlorotic than the parent ysl1/ysl3 double mutant line. The partial complementation observed is consistent with the strong overlap in expression of AtYSL1 and AtYSL3 in leaves (Waters et al., 2006). By contrast, AtYSL1 and AtYSL2 were completely unable to substitute for AtYSL3 with respect to reproductive function. This result implies that AtYSL3 has a distinct activity required for successful reproduction, which is not present in the closely related AtYSL1 and AtYSL2 proteins. It is tempting to speculate that this activity relates to AtYSL3’s apparent ability to transport Fe(III) in addition to Fe(II), but one could also speculate that failure to complement is the result of other transport properties that have not yet been determined, for example, the ability to transport Fe-NA complexes both into and out of cells.

MATERIALS AND METHODS:

Plant growth conditions
Seeds were surface sterilized in 70% ethanol with 0.05% TritonX-100 and then imbibed in sterile distilled water at 4C for 3-5 days. Plants were grown on sterile plates containing 1X MS medium. Plates were positioned vertically so that the roots grew along the surface rather than inside the agar, which allowed for easy transfer. Soil-grown plants were sown directly onto Metro-Mix (Sungro Horticulture, Bellevue, WA) treated with Gnatrol (Valent Biosciences, Walnut Creek, CA) to control fungus gnats. Growth chamber conditions for plates and soil-grown plants were 16 hours of light and 8 hours of dark at 22C.

Cloning of YSL3 cDNA
YSL3 cDNA was amplified by RT-PCR using Platinum Taq DNA Polymerase High Fidelity (Invitrogen) and the following primers: oAtYSL3c.39..65y (5’-ATCCACTAGTAA
CGGCGGaaatgaggagtatgatgagagag-3’), and oAtYSL3.4203..4198y (5’-CCGCC
ACTGTGCTGGGATctgtaactgatatattacggcat-3’). Each primer contains an 18
nucleotide (lower case) overlap with yeast expression vector pYES6/CT for in vivo homologous recombination. YSL3 cDNA and linearized pYES6/CT were co-transformed into Fe-uptake-defective yeast strain DEY1453 and recombinant plasmids were selected using blasticidin resistance.

Yeast functional complementation. Saccharomyces cerevisiae strain DEY1453 (MATa/MATa ade2/ADE2 can1/can1 his3/his3 leu2/leu2 trp1/trp1 ura3/ura3 fet3-2::HIS3/fet3-2::HIS3 fet4-1::LEU2/fet4-1::LEU2) was transformed with pGEV-Trp (Gao and Pinkham, 2000) together with pYES6/CT or pYES6/CT with AtYSL1 or AtYSL3 cDNA. For complementation assays SD –Trp media was made with Fe-free yeast nitrogen base buffered with 25 mM MES at pH 5.7 (for plates containing Fe(II)); or pH 6.0 (for plates containing Fe(III)). To prepare assay plates containing Fe(II), the following were added, in order, to the center of an empty plate: 125ul of 200mM of ascorbic acid, 7.5ul of freshly prepared Fe(II)SO4, and then 20 µl of 10mM NA. The solution was mixed briefly, and incubated at room temperature for 10 min to allow complex formation. Following this, 25 ml of molten SD –Trp with 10ug/ml Blasticidin was added to the plates, which were then allowed to solidify. In plates containing beta-estradiol, the stock solution was added to the molten agarose just prior to preparation of the plates. To prepare assay plates containing Fe(III), 34ul of 7.4mM FeCl3 and 25ul of 10mM DMA or 10 mM NA were placed in the center of an empty plate and incubated at room temperature for 10 minutes to allow complex formation. Following this, 25 ml of molten SD –Trp with 10ug/ml Blasticidin was added to the plates, which were then allowed to solidify. When preparing plates with BPDS, 25ul of 10mM BPDS was added just prior to the addition of molten medium. Nicotianamine was obtained from T. Hasegawa Company, LTD, Kawasaki-Shi, Japan)
Yeast suspensions were prepared from 3 day old yeast colonies. Colonies were removed from the plates, suspended in sterile H₂O, and the OD₅₅₀ of the resulting suspension was measured. The OD₅₅₀ of the suspension was brought to 0.1, serial dilutions (1:10, 1:100, 1:1000, and 1:10,000) of the suspension were then prepared, and 7 µl of each dilution was spotted on the plates. Plates were grown at 28°C for 3 days.

**Synchrotron X-ray Fluorescence Computed Microtomography**

Tomograms were collected at the bending magnet beamline X26A at the National Synchrotron Light Source, Brookhaven National Laboratory. X-ray fluorescence measurements were conducted using a 12 keV monochromatic x-ray beam, which was tuned using a Si (111) channel-cut monochromator. Monochromatic x-rays were focused to a beam size of 5 × 8 µm using Rh-coated, silicon Kirkpatrick-Baez microfocusing mirrors. Incident beam energy was monitored using an ion chamber upstream of the focusing optics. X-ray fluorescence spectra were collected with a Vortex-EX silicon-drift detector (SII Nanotechnology) with an active area of 50 mm². X-ray transmission through the sample was recorded simultaneously using a p-type, intrinsic, n-type (PIN) photodiode.

Individual seeds were attached to a 100 µm diameter silica fiber using Devcon® 5-minute epoxy resin, with the micropyle uppermost. The fiber was inserted in to a Huber 1001 goniometer, centered and mounted on a xyzθ stage. During fluorescence microtomography the seed samples were translated horizontally through the focused x-ray microbeam in step sizes ranging from 5-7 µm, and then rotated at intervals of between 0.8-1.1° angular steps and repeating the translation through a total of 180°. Full energy dispersive spectra were collected at each pixel with a dwell time of 2 seconds per pixel. Two dimensional sinograms (plot of intensity against θ) were computationally reconstructed using fast Fourier transform based Gridrec software developed by Brookhaven National Laboratory (Dowd et al., 1999), which is controlled by the Interactive Data
Language (IDL) programming software (Research Systems, Inc.) to provide images of the cross-sectional internal metal distribution.

Elemental abundances (weight fraction) were calculated for the fluorescence measurements, adapted from description by McNear et al. (McNear et al., 2005). Briefly, a thin-film standard reference material (SRM 1833) was measured prior to the collection of each data set to establish sensitivities (counts per second per µg cm⁻²) for Fe. We used an assumed object density of 1.2 g cm⁻³ for Arabidopsis seed, a measured voxel size of 3.887 × 10⁻⁸ cm³ (reconstructed pixel area × beam height) and the average Fe response from the sample to calculate the Fe content of a whole tomogram. This Fe abundance was used as a fixed value for input into the NRLXRF program (Naval Research Laboratory X-ray Fluorescence) (Criss, 1977) from which abundances for K, Ca, Mn, Ni, C and Zn were calculated. The concentration precision is typically ±15% and ±10% (1σ) for individual and mean values respectively.

**GFP fusion constructs and intracellular localization**

YSL1 cDNA was amplified by RT-PCR using Platinum Taq DNA Polymerase High Fidelity (Invitrogen) and the following primers: oAtYSL1c.2..27 (5'-CAGTCTCCATGGAA ATAGAGCAAAGA-3') and oAtYSL1c (5'-TCCTCTGAAGCTAAGAACTTCATACAT-3') which altered the stop codon from TAG to GAG. YSL1 cDNA was then introduced into the vector psmGFP (CD3-326, ABRC) to generate a C-terminal translational fusion to smGFP under the control of CaMV 35S promoter. The 35S::YSL1::GFP fusion construct was then transiently expressed in onion epidermal cells by biolistic bombardment. For onion bombardment, 6 mg gold microcarriers (1µm) were coated with 4 µg of DNA in the presence of 50 µL of 2.5 M CaCl₂ and 20 µL of 0.1 M spermidine, followed by washing with 70% ethanol and then resuspending in 100% ethanol. Gold microcarriers were placed on the macrocarriers and then bombarded onto onion epidermal peels on 1X MS medium plates at a distance of 6 cm using the PDS-1000/He instrument (Bio-Rad) with 1100 psi He pressure. The onion
epidermal peels were then incubated in the dark at 30°C. After 48 hours, samples were mounted in water for microscopic observation.

Full length YSL3 genomic DNA was amplified using Platinum Taq DNA Expand High-Fidelity polymerase (Roche) and the following primers: oAtYSL3.26..50 (5'-ACAGTCACATGAACCGGAATCTCGG-3'), and oAtYSL3.4208..4179 (5'-GTAACTCGAATATTTACTCGCATGAAGCC-3') which altered stop codon from TAA to TAC. YSL3 genomic DNA was then cloned into pDEST-GRuB (pPZP212 backbone with smGFP inserted using BamHI and EcoRI site) to generate C-terminal translational fusion to smGFP. The YSL3::GFP fusion construct was then stably expressed in ys1ysl3 double mutant plants following Agrobacterium-mediated transformation. Roots of 5 day old seedlings were mounted in water for microscopic observation. Green fluorescent cells were imaged by Zeiss 510 Meta Laser Scanning Confocal Microscope (Nikon) with excitation at 488 nm, and the fluorescence emission signal was recovered between 520 and 550 nm.

Inflorescence Grafting
The inflorescences (3-7 cm long, and with similar diameters) of soil-grown Col0 and ys1ysl3 plants were cut using a double-sided blade and then placed in water to prevent drying prior to grafting. A short tube (~0.5 - 1 cm) of appropriate diameter (Small Parts Inc., Part #: STT-20-10), was placed over the cut inflorescence stem of the stock, and the tube was filled with distilled water. The scions were then inserted into the other end of the tubing until they touched the stem of the scion. The plants were returned to trays covered with lids for 3-5 days to maintain the moisture, allowing plants to recover (Rhee and Somerville, 1995).

Mineral analysis
Metal ion contents of plant tissues were measured as described (Waters et al., 2006). For soil-grown plants, leaf samples were collected 20 days after sowing.
Quantitative RT-PCR
RNA isolation and reverse transcriptase reactions were performed as described (Waters et al., 2006). Quantitative real-time PCR was performed in a 25-µL reaction volume containing 25 ng (based on the initial input of RNA to the RT reaction) cDNA, 400 nM each gene-specific primers, and Brilliant® II SYBR® Green QPCR Master Mix (Stratagene). Primer sequences used were Actin2: oAtActin2.qPCR FW (TCTTCCGCTCTTTCTTTCCAAGC) and oAtActin2.qPCR REV (ACCATTGTACACACGATTGTTG); EF-1a.qPCR FW (GCACGCTCTTTGCTTTCAACC) and EF-1a.qPCR REV (GGGTGGTGGCATCATCCATTTGTTAC); YSL3: oAtYSL3.qPCR FW (GTTACAGCATTGCAGTTGGAGGTG) and oAtYSL3.qPCR REV (AAGCGGTCATCCAAACGGATTTCC). The two-step thermal cycling profile used was 15 min at 95°C, followed by 40 cycles of 10 sec at 95°C, 30 sec at 60°C. Following amplification, melt curves were performed to verify that a single product was amplified.

The final threshold cycle (Ct) values were the mean of three replicates. The comparative ΔΔCt method was used to analyze the results (User Bulletin 2; Applied Biosystems PRISM sequence detection system). Normalization of the values was based on Actin2 and EF-1a. A negative control with water instead of template was included for each qRT-PCR reaction set.

Overexpression of YSL3
Whole YSL3 genomic DNA was amplified using Platinum Taq DNA Polymerase High Fidelity (Invitrogen) and the following primers: oAtYSL3.26..50 (5'-ACAGTCACATGAACCAGAATCTCGG-3'), and oAtYSL3.4209..4175 (5'-TTAACTCGAATATTTAATCCTCGGAGAAGGCATCGAAGCCCATAC-3'). YSL3 genomic DNA was cloned into vector pMN20 (Weigel et al., 2000), containing four copies of the CaMV 35S enhancer. The construct was stably transformed into wild type Arabidopsis plants and homozygous transformants were obtained by kanamycin selection.
Chlorophyll content determination
For Fe starvation tests, YSL3-overexpression lines were grown on 1X MS medium for 10 days and then transferred to MS-Fe medium for a variable number of days prior to quantification of chlorophyll. Leaves of soil-grown YSL3p::YSL1 and YSL3p::YSL2 lines were collected at day 20. Shoots and leaves were placed in 1 mL of $N,N'$-dimethylformamide and chlorophyll was extracted overnight. Total chlorophyll was determined as described (Inskeep and Bloom, 1985).

Germination Tests
Seeds were soaked in 0.1% agarose to imbibe at 4°C for 72h. One hundred seeds were plated onto soil. Percentage viability of seeds was determined 7 days after sowing, at which time germination was scored as successful emergence of the hypocotyls and cotyledons.

Collection and Analysis of Phloem Exudates
Phloem exudates were collected from adult rosette leaves of 25-day-old soil-grown Arabidopsis plants at the start of flowering, using procedures previously described (Fan et al., 2009). The third and fourth leaves were cut and then the tip of the petiole was recut in EDTA buffer (5mM Na2EDTA, pH 7.5, osmotically adjusted to 270 mosmol with sorbitol). Leaves were washed with a large volume of sterile EDTA buffer to remove contaminants and then placed in 200 ul of fresh EDTA buffer. During phloem sap exudation, the leaves were illuminated (25 µE) and incubated at 22C. After 1 hour of bleeding, the buffer solution containing phloem exudates was analyzed for sugar content. Sucrose and glucose content were measured by the DNS method (Bernfeld, 1955).

Complementation of ysl1ysl3 double mutants
The YSL3 promoter was amplified using Platinum Taq DNA Polymerase High Fidelity (Invitrogen) and the following primers: oAtYSL3.26..50 (5' - ACAGTCACATGAACCGGAA TCTCGG-3'), and oAtYSL3.1521..1496 (5' - ACTCCTCAGTTTTTCCAAGAACGA-3'). The YSL3 promoter was then cloned
into vector pPZP222. For cloning of YSL1 and YSL2 cDNA, an HA tag (YPYDVPDYA) was added to the C terminus of each cDNA in a two step strategy. YSL1 cDNA was amplified by RT-PCR using Platinum Taq DNA Polymerase High Fidelity (Invitrogen) and the following primers: oAtYSL1.1526..1555 (5' - CAGTCTCCATGGAAATAGAGCAAAGAAGG - 3'), and oAtYSL1.HA1 (5' - GTCGTACCGGG TATGAAGCTAAGAACTTCATACATATCGGAGG - 3') which altered the stop codon from TAG to TAC and added the first twelve nucleotides of the HA tag to the 3' end of the YSL1 cDNA. A secondary PCR reaction was then performed using primary PCR product as template and the following primers: oAtYSL1.1526..1555 (5' - CAGTCTCCATGGAAATAGAGCAAAGAAGG - 3'), and oAtYSL1.HA2 (5' - TCACGCGTAGTCCGGCAGTACGGAAGCTAAGAACTTCATACATATCGGAGG - 3') which adds the entire HA tag sequence to the 3' end of the YSL1 cDNA. YSL2 cDNA was amplified using the same method as for YSL1 cDNA. Primers for YSL2 primary PCR were oAtYSL2.1541..1567 (5' - CTTCAAATGGAAAACGAAAGGGTTGAG - 3'), and oAtYSL2.HA1 (5' - GTCGTACCGGGTATGAGCCGCAGTGAAGTTCATACAGCAGT - 3'). Primers for YSL2 secondary PCR were oAtYSL2.1541..1567 (5' - CTTCAAATGGAAAACGAAAGGGTTGAG - 3'), and oAtYSL2.HA2 (5' - TCACGCGTAGTCCGGCAGTACGGAAGCTAAGAACTTCATACAGAT - 3'). YSL1 and YSL2 cDNA were cloned into the vector YSL3p/pPZP222 under the control of the YSL3 promoter. YSL3p::YSL1 and YSL3p::YSL2 constructs were then stably transformed into ysl1 ysl3 double mutant and selected using gentamycin resistance.

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FIGURE LEGENDS

Figure 1. Functional complementation of fet3fet4 yeast. DEY1453-derived yeast strains transformed with pGEV-TRP and constructs containing AtYSL1, AtYSL3, OsYSL15 or ZmYS1 cDNA, or the empty pYES6/CT vector, were grown on synthetic defined medium containing variable conditions for iron (Fe(II) or Fe(III)), chelator (DMA or NA), and beta-estradiol (BE). Pairs of spots correspond to 10-fold and 100-fold dilutions of the original cultures.
(A) Results using Fe(II) and NA. Each plate contained the constituents indicated, as the following concentrations: 50 µM iron citrate, 3uM Fe(II)SO4, 8 µM NA, 10 nM BE.
(B) Results using Fe(III) and PS. Each plate contained the constituents indicated, as the following concentrations: 50 µM iron citrate, 10uM Fe(III)Cl3, 10uM DMA, 40nM beta-estradiol. In addition, the Fe(II) chelator, BPDS, was added to some plates, as indicated.
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Figure 2. Synchrotron X-ray Fluorescence Computed Microtomography showing the distribution and abundance (in µg g⁻¹) of Fe. Tomograms (virtual cross sections) were collected via SXRF computed microtomography from intact, dry Arabidopsis seed. In each panel, the embryonic radicle is at the left, and the cotyledons are at the right. A single provascular strand is visible in the radicle and three provascular strands are visible in each cotyledon.
(A) Wild type Columbia-0 (Col-0) Scale bar is 100 µm.
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Figure 3. Localization of AtYSL1 and AtYSL3 using GFP. (A-F) Localization in onion epidermal cells using microprojectile bombardment. (G-L) Localization in stably transformed Arabidopsis. 15 optical sections were fused to produce each micrograph.

(A) Fluorescence image of onion cell bombarded with 35S-YSL1-GFP construct.
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(C) Overlay of images shown in (A) and (B)
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(F) Overlay of images shown in (D) and (E)
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(H) DIC image of the root shown in (G).
(I) Overlay of images shown in (G) and (H)
(J) Fluorescence image of the root of a plant stably transformed with a 35S-TIP construct. Arrows indicate positions where the fluorescence signal deviates around nuclei.
(K) DIC image of the root shown in (J).
(L) Overlay of images shown in (J) and (K).

Figure 4. Inflorescence grafting experiment. ysl1ysl3 double mutant scions (inflorescences) grafted onto WT stocks are labeled as ysl1ysl3+WT. WT+WT indicates wild type plants that were self-grafted as positive controls. ysl1ysl3 double mutant was used as negative control since it was not possible to generate
self-grafted *ysl1ysl3* plants. Error bars represent standard deviation. Asterisks indicate $P < 0.05$ by T-test.

(A) Average seed number per silique. Each silique on the grafted inflorescences was opened and the seed number was counted. WT+WT and *ysl1ysl3* contain 4 replicates. *ysl1ysl3+WT* contains 6 replicates.

(B) Average weight of an individual seed produced by the grafted inflorescences. WT+WT and *ysl1ysl3* contain 4 replicates. *ysl1ysl3+WT* contains 6 replicates.

(C) The percentage of seeds germinating on soil was determined. Four replicate batches of 100 of each seed type were used in the analysis. Germination was defined as emergence of the radical, and was scored every 24 hours after plating.

Figure 5. Metal concentration of seeds of grafted plants. Metal content of the seeds produced by un-grafted WT plants, by WT self-grafted plants, by *ysl1ysl3* inflorescences grafted to WT rosettes, and by *ysl1ysl3* un-grafted plants was determined using ICP-MS. As a control for the effect of inflorescence grafting on seed metal concentration, seeds from un-grafted WT plants were also measured. WT+WT and *ysl1ysl3* contain 4 replicates; *ysl1ysl3+WT* contains 6 replicates; un-grafted WT contains 10 replicates. Asterisks indicate $P < 0.05$ by T-test.

Figure 6. Sucrose levels in the phloem of WT and *ysl1ysl3* double mutant plants.

Figure 7. *AtYSL3* enhanced expression lines YSL3 OX L5.8, YSL3 OX L7.1, and YSL3 OX L13.2. Each line contains a 4X35S enhancer sequence upstream of a *AtYSL3* genomic clone containing 1487 bp of native *AtYSL3* sequence upstream of the initiating ATG.

(A-D) Expression of *AtYSL3* by quantitative RT-PCR. Lane1: Col0. Lane2: YSL3 OX L5.8. Lane3: YSL3 OX L7.1. Lane4: YSL3 OX L13.2.

(A) Leaves.

(B) Flowers.

(C) Flower buds.
(D) Cauline leaves.

(E) ICP-MS determination of Mn, Fe, Cu, and Zn concentrations of leaves from 20 day old soil-grown plants. Results are given as ppm. Error bars represent standard error. Each sample contains 10 replicates. Asterisks indicate P < 0.05 by T-test.

(F) ICP-MS determination of Mn, Fe, Cu, and Zn concentrations of seeds. Results are given as ppm. Error bars represent standard error. Each sample contains 10 replicates. Asterisks indicate P < 0.05 by T-test.

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(A) 20 day old soil-grown Col0 WT, YSL3p::YSL1, YSL3p::YSL2, and ysl1ysl3 plants.

(B) Total chlorophyll concentration of leaves of Col0 WT, YSL3p::YSL1, YSL3p::YSL2, and ysl1ysl3 grown on soil for 20 days. Each sample represents 10 replicates.

(C) Total chlorophyll concentration of leaves of Col0 WT, YSL3::GFP, and ysl1ysl3 grown on soil for 20 days.

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Supplemental Figure 1. The sequence of the protein encoded by the AtYSL3 cDNA. Residues 289-338 of the AtYSL3 protein are shown. One change relative to the sequence of Col0 At5g53550 was found in the cDNA that resulted in the change of a lysine residue to an arginine residue (shown at top). For comparison, the sequences of all other Arabidopsis and rice YSLs are shown. At bottom, “*” indicates perfect conservation; “:” indicates that conserved substitutions are observed; “.” indicates that semi-conserved substitutions are observed.

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