The Arabidopsis AtOPT3 Protein Functions in Metal Homeostasis and Movement of Iron to Developing Seeds

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The Arabidopsis thaliana AtOPT3 belongs to the oligopeptide transporter (OPT) family, a relatively poorly characterized family of peptide/modified peptide transporters found in archebacteria, bacteria, fungi, and plants. A null mutation in AtOPT3 resulted in embryo lethality, indicating an essential role for AtOPT3 in embryo development. In this article, we report on the isolation and phenotypic characterization of a second AtOPT3 mutant line, opt3-2, harboring a T-DNA insertion in the 5′ untranslated region of AtOPT3. The T-DNA insertion in the AtOPT3 promoter resulted in reduced but sufficient AtOPT3 expression to allow embryo formation in opt3-2 homozygous seeds. Phenotypic analyses of opt3-2 plants revealed three interesting loss-of-function phenotypes associated with iron metabolism. First, reduced AtOPT3 expression in opt3-2 plants resulted in the constitutive expression of root iron deficiency responses regardless of exogenous iron supply. Second, deregulation of root iron uptake processes in opt3-2 roots resulted in the accumulation of very high levels of iron in opt3-2 tissues. Hyperaccumulation of iron in opt3-2 resulted in the formation of brown necrotic areas in opt3-2 leaves and was more pronounced during the seed-filling stage. Third, reduced AtOPT3 expression resulted in decreased accumulation of iron in opt3-2 seeds. The reduced accumulation of iron in opt3-2 seeds is especially noteworthy considering the excessively high levels of accumulated iron in other opt3-2 tissues. AtOPT3, therefore, plays a critical role in two important aspects of iron metabolism, namely, maintenance of whole-plant iron homeostasis and iron nutrition of developing seeds.

Plants, as sessile organisms, are restricted to their habitats, creating problems when nutritional conditions become limiting. To cope with nutrient deficiencies, higher plants possess a variety of responses to both their internal nutritional status and to the external availability of nutrients. Iron (Fe) is an essential nutrient for plant growth and is often limited in soils, especially those with high pH (calcareaous; Shenker and Chen, 2005). Plants have evolved two strategies to efficiently take up Fe from the soil (for recent reviews, see Kerkeb and Connolly, 2006; Kim and Guerinot, 2007). Graminaceous plants utilize strategy I for Fe uptake, where Fe chelators, phytosiderophores (PSs), are excreted from roots and the Fe-PS complex is transported into the plant via the YELLOW STRIPE1 (YS1) protein (Takagi et al., 1984; Von Wieren et al., 1994; Curie et al., 2001; Yen et al., 2001). The maize (Zea mays) ZmYS1 was shown by expression in yeast and Xenopus laevis oocytes to function as a proton-coupled symporter for PS-chelated metals (Curie et al., 2001; Roberts et al., 2004; Schaaf et al., 2005). Plant PSs are composed of mugeneic acids (MAs), all derived from nicotianamine (NA). NA, unlike PS, is not secreted and is synthesized by all vascular plants. The ability to enzymatically convert NA to MAs is unique to graminaceous plants (Takahashi et al., 1999; Bashir et al., 2006).

Dicots and nongraminaceous monocots utilize strategy II for Fe uptake, which involves proton extrusion to solubilize Fe³⁺ in the soil and a plasmalemma root Fe(III)-chelate reductase (FRO) to reduce Fe³⁺ (Robinson et al., 1999). The resulting Fe²⁺ is transported via the IRT1 transporter (Eide et al., 1996; Henriques et al., 2002; Varotto et al., 2002; Vert et al., 2002). In Arabidopsis (Arabidopsis thaliana), a member of the H⁺-ATPase family likely mediates proton efflux and AtFRO2 is responsible for the reduction of Fe³⁺. To avoid the toxic effects of excess Fe accumulation, Fe acquisition in strategy I and strategy II plants is tightly regulated and is responsive to Fe availability. For example, biosynthesis of MAs in strategy I plants and expression of FRO and IRT1 in strategy II plants are up-regulated only under Fe-limiting conditions (Nakanishi et al., 1993; Okumura et al., 1994; Eide et al., 1996; Robinson et al., 1999; Takahashi et al., 1999; Bashir et al., 2006).
ZmYS1 belongs to the oligopeptide transporter (OPT) family, a relatively poorly characterized family of transport proteins involved in the transport of peptides and amino acid-derived compounds. OPT transporters are found in archaeabacteria, bacteria, fungi, and plants, but not in animals (Saier, 2000; Yen et al., 2001; Stacey et al., 2002a; Waterworth and Bray, 2006; Tsay et al., 2007). Curie et al. (2001) utilized the ZmYS1 gene sequence to identify eight putative orthologs in Arabidopsis (YSI-like genes, YSLS). A rice (Oryza sativa) OsYSL2 gene was also identified, one of 19 putative YSL genes in rice (Koike et al., 2004; Le Jean et al., 2005). Several lines of evidence indicate that YSLS are involved in the intercellular transport of Fe chelates, specifically Fe(II)-NA complexes. For example, expression of ZmYS1 (Schäaf et al., 2005) and OsYSL2 (Koike et al., 2004) in Xenopus oocytes conferred the ability to take up Fe(II)-NA, as well as other metal-NA chelates. The Thlaspi caerulescens TcYSL3 and the Arabidopsis AtYSL2 were also shown to mediate the transport of Fe-NA complexes (Gendre et al., 2007). OsYSL2 is expressed in the companion cells of the phloem and in developing seeds, suggesting a role for OsYSL2 in the transport of metal-NA complexes in the phloem and their unloading into the grain (Koike et al., 2004). In Arabidopsis, Le Jean et al. (2005) reported that loss-of-function mutations in AtYSL1 resulted in increased accumulation of NA in shoots and a concomitant decrease in Fe and NA accumulation in seeds of ysl1 plants compared with wild-type plants. Waters et al. (2006) also reported that mutations in both AtYSL1 and AtYSL3 resulted in reduced Fe accumulation in leaves and seeds compared with the wild type. The double mutant also exhibited interveinal chlorosis and reduced fertility due to defective anther and embryo development. These results indicate that YSL proteins are important in the transport of chelated Fe, most likely Fe(II)-NA, especially to developing seeds.

Members of the OPT family were first characterized in yeast. Yeast OPTs were shown to transport tetra- and pentapeptides (Lubkowitz et al., 1997, 1998; Hauser et al., 2000; Osawa et al., 2006), as well as longer oligopeptides of up to eight amino acids in length (Reuss and Morschhauser, 2006). Yeast OPTs were also shown to mediate the transport of the modified peptide glutathione (γ-Glu-Cys-Gly; Bourbouloux et al., 2000; Osawa et al., 2006). We identified nine OPT (AtOPT1–AtOPT9) proteins in Arabidopsis by database searches for homology to the Candida albicans CaOPT1p (Koh et al., 2002). Growth complementation assays in yeast demonstrated that five (AtOPTs 1, 4, 5, 6, and 7) of the AtOPTs were functional peptide transporters, transporting at least one of the synthetic peptides KLLG, KGLL, and KLLLG (Koh et al., 2002). We recently showed by two-electrode voltage-clamp studies in Xenopus oocytes that the Saccharomyces cerevisiae ScOPT1p and the Arabidopsis AtOPT4 are proton-coupled transporters with broad but distinct substrate specificities and affinities (Osawa et al., 2006).

The AtOPTs and AtYSLS represent two subfamilies of OPT transporters in Arabidopsis (Saier, 2000; Yen et al., 2001; Stacey et al., 2002a; Supplemental Fig. S1). These two subfamilies show significant sequence divergence (i.e. are only 10%–16% similar at the protein level), with the AtOPTs being more phylogenetically related to yeast OPTs (i.e. 34%–37% similarity to CaOPT1p). Until recently, our data suggested that the AtOPTs transport small peptides for nutrition (Koh et al., 2002; Osawa et al., 2006). However, we found that the expression of a transcriptional AtOPT3-GUS (β-glucuronidase) fusion is up-regulated under Fe-limiting conditions (Stacey et al., 2006), consistent with the earlier findings of Wintz et al. (2003). These data suggested a possible function for AtOPT3 in the transport of Fe chelates similar to AtYSL proteins. AtOPT3 is essential for embryo development as indicated by the embryo-lethal phenotype associated with an AtOPT3 T-DNA mutation (opt3-1; Stacey et al., 2002b). The embryo lethality of the opt3-1 mutant precluded us from further elucidating the possible function of AtOPT3 in Fe metabolism.

In this article, we report the isolation and characterization of a second AtOPT3 mutant, opt3-2, harboring a T-DNA insertion in the AtOPT3 promoter region. The T-DNA insertion resulted in reduced but sufficient expression of AtOPT3 in homozygotes to allow embryo formation. Subsequent phenotypic analyses of the mutant showed that opt3-2 roots exhibited Fe starvation phenotypes even when grown under Fe-sufficient conditions and, more importantly, even when in planta Fe levels were excessively high. We also found that reduced AtOPT3 expression in the mutant resulted in decreased Fe accumulation in opt3-2 seeds. These data indicate that transport mediated by AtOPT3 may be a component of a signal transduction mechanism regulating Arabidopsis root Fe deficiency responses. Our data also indicate that AtOPT3, like AtYSLS, is critical for the mobilization of Fe to developing seeds, suggesting a function for AtOPT3 in phloem and/or postphloem transport processes.

RESULTS

Identification of a Nonlethal AtOPT3 Mutant Allele

We previously reported two important findings concerning the function of AtOPT3 in plant growth and development. First, a T-DNA insertion in the fourth exon of AtOPT3 resulted in embryo lethality (opt3-1; Stacey et al., 2002b). Second, AtOPT3 is expressed in vascular tissues and developing embryos and its expression is up-regulated under Fe-limiting conditions (Stacey et al., 2006). The embryo lethality of the opt3-1 mutation precluded further elucidation of the functional role of AtOPT3 in plant development, for example, in Fe transport. To identify weaker opt3 alleles, we characterized five AtOPT3 TILLING lines (http://tilling.fhcrc.org:9366/). Unfortunately, none of these lines showed any observable phenotype that cosegregated with the opt3 mutations (data not shown). We subsequently obtained a mutant line, opt3-2, harboring a T-DNA insertion 36 bp upstream.
of the AtOPT3 start codon (Fig. 1A). To determine if the T-DNA insertion affected the level of AtOPT3 expression, we performed semiquantitative reverse transcription (RT)-PCR on total RNA isolated from selected opt3-2 homozygotes and from the ecotype Columbia-0 of Arabidopsis (Col-0; wild type). We found that the accumulation of AtOPT3 transcripts in the wild type was up-regulated in both shoots and roots by limiting Fe (Fig. 1B). This is consistent with the previously reported Fe regulation of AtOPT3 expression (Wintz et al., 2003; Stacey et al., 2006). Compared with Col-0, opt3-2 exhibited reduced, but detectable, accumulation of AtOPT3 transcripts under both Fe-sufficient and Fe-deficient conditions (Fig. 1B). Also, no detectable up-regulation of AtOPT3 expression by limiting Fe was observed in the opt3-2 mutant (Fig. 1B).

When grown under Fe-sufficient conditions, opt3-2 seedlings were not chlorotic but were slightly smaller than Col-0 seedlings (Fig. 2A). Consistent with the lack of obvious chlorosis in opt3-2, chlorophyll content of mutant seedlings was comparable to that of Col-0 (data not shown). When grown under Fe-deficient conditions, both Col-0 and opt3-2 seedlings developed similar degrees of leaf chlorosis (Fig. 2A) and had similar chlorophyll content (data not shown). To determine if opt3-2 is deficient in its ability to accumulate Fe, we utilized Perl’s stain to localize Fe$^{3+}$ in Col-0 and opt3-2 tissues. Interestingly, we found that opt3-2 seedlings grown under Fe-sufficient conditions showed strong Fe$^{3+}$ staining in leaves and roots, mostly in vascular tissues and trichomes. In contrast, no detectable or weak Fe$^{3+}$ staining was observed in Col-0 leaves and roots, respectively (Fig. 2B). Altered distribution of Fe$^{3+}$ was observed only in opt3-2 homozygotes but not in heterozygotes, indicating that the mutation is recessive (data not shown).

Aberrant development of chloroplasts and leaf palisade parenchyma cells was observed in Fe transport mutants of Arabidopsis (Henriques et al., 2002; Duy et al., 2007). To determine if palisade parenchyma cells in opt3-2 leaves were developmentally compromised, we microscopically examined thin sections of wild-type and opt3-2 leaf tissues. In the wild type, the subepidermal layer of palisade parenchyma cells appeared columnar and was distinguishable from the underlying spongy parenchyma cells (Fig. 2C). In contrast, palisade parenchyma cells in opt3-2 leaves, like in the iirt mutant, failed to elongate and remained indistinguishable from the underlying spongy parenchyma cells (Fig. 2C). Unlike iirt1 shoots, opt3-2 shoots were not chlorotic (Fig. 2A) and synthesized wild-type levels of chlorophyll (data not shown). The failure of palisade parenchyma cells to develop normally in opt3-2 leaves suggests a critical role for AtOPT3 function in the differentiation of palisade cells during leaf development.

Taken together, these data indicate that the opt3-2 line expresses a sufficient amount of AtOPT3 to allow embryo formation in opt3-2 homozygous seeds. However, the significantly reduced expression of AtOPT3 in opt3-2 plants resulted in observable loss-of-function phenotypes associated with Fe metabolism, i.e. altered distribution of Fe$^{3+}$ and aberrant development of leaf palisade parenchyma cells.

**Constitutive Expression of Fe Deficiency Responses in opt3-2 Plants**

The aberrant acquisition and distribution of Fe$^{3+}$ in opt3-2 seedlings prompted us to examine if opt3-2 plants were compromised in their regulation of root Fe deficiency responses. We assayed the expression of Fe(III)-chelate reductase (FRO2) activity in Col-0 and opt3-2 seedlings grown under Fe-sufficient and Fe-deficient conditions (Fig. 3). We found that FRO2 activity in opt3-2 was constitutively expressed regardless of Fe availability (Fig. 3A). Co-segregation analysis of the constitutively expressed Fe(III)-chelate reductase phenotype showed that the phenotype was observed only in homozygous plants but not in heterozygotes, again indicating that the opt3-2 mutation is recessive (data not shown). We also examined the pattern of IRT1 expression in opt3-2 plants in response to exogenous Fe supply by semiquantitative RT-PCR. Like FRO2, IRT1 expression in opt3-2 roots was constitutively expressed regardless of Fe status in the growth medium (Fig. 3B). Consistent with previous reports (Grusak et al., 1993; Robinson et al., 1999; Vert et al., 2003), FRO2 and IRT1 expression in wild-type roots was up-regulated by Fe-deficient conditions (Fig. 3, A and B). Constitutive expression of FRO2 and AtIRT1 in opt3-2 roots was unexpected because opt3-2 plants did not exhibit obvious Fe deficiency phenotypes, e.g. were not chlorotic and actually accumulated high levels of Fe$^{3+}$ in both shoots and roots (Fig. 2B).

Constitutive expression of root Fe deficiency responses in the Arabidopsis frd3 and tomato (Solanum

**Figure 1.** Isolation of a T-DNA insertion mutation in the promoter of AtOPT3. A, Diagram of the T-DNA and insertion site upstream of the AtOPT3 gene in opt3-2. The T-DNA is inserted 36 bp upstream of the AtOPT3 start codon in the orientation indicated. Boxes represent exons and lines represent introns. RB, Right border; LB, left border. Bar = 500 bp. B, Semiquantitative determination of AtOPT3 expression in Col-0 and opt3-2 by RT-PCR. Plants were grown on Murashige and Skoog plates for 15 d then grown for 4 d under Fe-sufficient (+) or Fe-deficient (−) conditions. Total RNA was isolated from roots and shoots and RT-PCR amplification of Actin2 is also shown.
lycopersicum) chloronerva (chlhn) mutants was reported to be due to reduced availability of intracellular Fe in mutant shoots (Pich et al., 2001; Rogers and Guerinot, 2002; Green and Rogers, 2004). To determine if intracellular accumulation of Fe is compromised in opt3-2 plants, we examined the accumulation of AtFERRITIN1 (AtFER1) transcripts in wild-type and opt3-2 plants in response to exogenous Fe supply. Ferritins are Fe-storage proteins that are critical in Fe homeostasis and are expressed in response to increased levels of cellular Fe (Briat et al., 1999; Petit et al., 2001). We found that AtFER1 expression in shoots and roots of wild-type plants was Fe regulated as indicated by the increased levels of AtFER1 transcripts under Fe-sufficient conditions (Fig. 3B). Similar Fe-regulated expression of AtFER1 was observed in opt3-2 shoots, whereas no reduction in AtFER1 expression was observed in opt3-2 roots under Fe-deficient conditions (Fig. 3B). These results, together with the lack of detectable chlorosis in opt3-2 plants, indicate that deregulation of root Fe...
deficiency responses in opt3-2 is unlikely due to a gross defect in the intracellular accumulation of Fe in opt3-2 cells.

Genetic Complementation of opt3-2

To confirm that the defective Fe acquisition phenotypes described above were indeed due to the AtOPT3 lesion, genetic complementation was performed by transforming opt3-2 plants with the AtOPT3 gene expressed from the AtOPT3 promoter (promoter::AtOPT3; Stacey et al., 2002b). As a control, transformation was also performed with a DNA encoding the GUS gene expressed from the native AtOPT3 promoter (promoter::GUS). Five independent transgenic lines expressing either promoter::AtOPT3 or promoter::GUS were assayed for FRO2 activity. As shown in Figure 4A, increased FRO2 activity in plants expressing promoter::AtOPT3 was observed only under Fe-deficient conditions. In contrast, plants expressing promoter::GUS showed constitutively high Fe(III)-chelate reductase activity regardless of Fe availability in the growth medium (Fig. 4B). When stained for Fe(III), lines expressing promoter::AtOPT3 gave wild-type levels of Fe(III), whereas lines expressing promoter::GUS exhibited the characteristic altered Fe(III) distribution phenotype of opt3-2 (data not shown). We also examined FRO2 activity and Fe(III) accumulation in several independent transgenic lines overexpressing AtOPT3 from the constitutively expressed CAMV35S. This was done to determine if the aberrant Fe acquisition exhibited by opt3-2 plants was due to ectopic AtOPT3 expression, arising from the T-DNA insertion in the promoter region. We found that none of the transgenic lines exhibited constitutive FRO2 activity nor accumulated excess Fe(III) (data not shown). Taken together, these results indicate that the observed mutant phenotypes are due to the strong down-regulation of AtOPT3 expression in the opt3-2 mutant. This is also indicated by the cosegregation of the deregulated FRO2 activity phenotype with opt3-2 homozygotes (data not shown).

Overaccumulation of Metals in opt3-2 Plants

The high level of stainable Fe(III) in opt3-2 seedlings prompted us to examine the levels of accumulated macro- and micronutrients in adult opt3-2 and wild-type plants by inductively coupled plasma optical emission spectroscopy. Consistent with the high stainable Fe(III) in opt3-2 seedlings, Fe concentration in shoots of opt3-2 plants (478 μg g⁻¹ Fe) at bolting was 8.4-fold higher than in Col-0 (59 μg g⁻¹ Fe; Fig. 5A). In addition to Fe, manganese (Mn), zinc (Zn), and magnesium (Mg) concentrations in opt3-2 were also significantly higher than in Col-0 by at least 2-fold (Fig. 5A). No major differences were observed in copper (Cu), calcium (Ca), potassium (K), and phosphorus (P) concentration in Col-0 and opt3-2 tissues (Fig. 5). We also examined the mineral concentration in rosette leaves, inflorescence stems, and siliques of opt3-2 and Col-0 plants at 10 d after bolting. Consistent with the overaccumulation of Fe in opt3-2 plants at the bolting stage, we found that opt3-2 accumulated significantly higher amounts of Fe in rosette leaves (Fig. 6A), inflorescence stems (Fig. 6B), and siliques (Fig. 6C) compared with corresponding wild-type tissues. We also found that hyperaccumulation of Fe in opt3-2 tissues was more pronounced at later stages of seed filling. For example, Fe concentration in rosette leaves (1,099 μg g⁻¹) and siliques (250 μg g⁻¹) of opt3-2 was 28-fold and 6-fold higher than in corresponding Col-0 tissues, respectively (Fig. 6, A and C). Inflorescence stems of opt3-2 did not accumulate as much Fe as in rosette leaves and siliques. However, Fe concentration in opt3-2 inflorescence stems (16 μg g⁻¹ Fe) was still 2.6-fold higher than in wild-type inflorescence stems (Fig. 6B). Significantly higher concentrations of Mn, Zn, and Cu were also accumulated in opt3-2 rosette leaves, inflorescence stems, and siliques compared with corresponding Col-0 tissues (Fig. 6).

Leaves of opt3-2 showed necrotic lesions (Fig. 7, A and B), which were absent in Col-0 leaves (Fig. 7C). Lesions were observed only in older rosette and cauline leaves.
but not in newly formed leaves (data not shown). When stained for Fe$^{3+}$, Col-0 leaves showed no detectable Fe$^{3+}$ staining (Fig. 7D). In contrast, opt3-2 rosette leaves (Fig. 7E) and older cauline leaves (data not shown) showed high levels of stainable Fe$^{3+}$ at discrete spots, mostly where trichomes were localized (Fig. 7, E and F). Higher accumulation of Fe$^{3+}$ was also observed along leaf margins (Fig. 7E). Necrotic lesions in opt3-2 leaves mostly coincided with areas that stained strongly for Fe$^{3+}$ (data not shown) and were likely symptoms of metal toxicity.

Reproductive tissues of opt3-2 also showed significantly higher levels of Fe$^{3+}$ accumulation compared with corresponding wild-type tissues. Similar to leaves, no detectable Fe$^{3+}$ was observed in Col-0 flowers (Fig. 7G). In contrast, opt3-2 flowers showed strong Fe$^{3+}$ staining in sepals (Fig. 7, H and I) and the stamen vascular bundle (Fig. 7J). No detectable Fe$^{3+}$ staining was observed in wild-type siliques (Fig. 7, K-M) or in young opt3-2 siliques (stage 16; Fig. 7I). Distal ends of opt3-2 siliques at harvest appeared shriveled and discolored (Fig. 7Q) when compared with corresponding wild-type siliques (Fig. 7R). Like the necrotic lesions in opt3-2 leaves, discoloration and shriveled distal ends of opt3-2 siliques were likely due to high, toxic levels of accumulated Fe.

In summary, opt3-2 accumulated high levels of Fe in leaves, flowers, and siliques as determined quantitatively by micronutrient analysis and qualitatively by Fe$^{3+}$ staining. Fe in opt3-2 leaves accumulated at toxic levels, such that necrotic lesions were observed at sites where Fe$^{3+}$ was localized, mainly in trichomes and tissues immediately surrounding trichomes. Fe$^{3+}$ in opt3-2 siliques accumulated mostly in stigmas, in styles and in vascular tissues and distal ends of pod walls. A significant portion of the accumulated Fe in opt3-2 siliques remained in seed pods, as indicated by high levels of stainable Fe$^{3+}$ in these tissues at seed harvest. Lastly, concentrations of Mn, Zn, and Cu in opt3-2 tissues was also higher compared with that in wild-type tissues.

Reduced Seed Fe Concentration and Seed Yield in opt3-2 Plants

To determine if the opt3-2 mutation affected the ability of mutant plants to mobilize Fe to developing seeds, we determined the Fe concentration in mutant seeds and compared it with that in wild-type seeds. We found that opt3-2 seeds had 35% less Fe compared with the wild type (Fig. 8A). In contrast to Fe, opt3-2 seeds contained significantly higher levels of Mn, Zn, and Cu than wild-type seeds (Fig. 8A). This is consistent with the higher levels of these micronutrients in other opt3-2 tissues analyzed (Figs. 5 and 6). No significant difference was observed in Ca, K, Mg, and P concentration in wild-type and opt3-2 seeds (Fig. 8B). When stained for Fe$^{3+}$ for 30 min, wild-type seeds showed staining in the developing vasculature of embryo axis and cotyledons (Fig. 9, A and B), consistent with a recently published report on the localization of stored Fe in mature seed.

**Figure 5.** Metal and macronutrient concentrations in Col-0 and opt3-2 plants at bolting. Plants were grown in soil until bolting, then aerial tissues from 16 plants were harvested, pooled, and analyzed for metal (A) and macronutrient (B) concentration. Means and se obtained from three biological replicates are shown.

**Figure 6.** Overaccumulation of Fe, Mn, Zn, and Cu in opt3-2 plants at 10 d after bolting. Rosette leaves (A), inflorescence stems (B), and siliques (C) were harvested at 10 d after bolting and analyzed for metal concentration. Samples represent pooled tissues from 16 plants. At 10 d after bolting, very few flower buds remained and siliques were not yet dehiscent. Inflorescence stems included cauline leaves and a few remaining flowers and excluded siliques that were detached and assayed separately. Means and se obtained from three biological replicates are shown.
Arabidopsis seeds (Kim et al., 2006). In contrast, no detectable Fe$^{3+}$ was observed in opt3-2 embryos (Fig. 9, A and B) under similar staining conditions, although weak Fe$^{3+}$ staining in developing embryonic vascular tissues was detected after a longer staining period (i.e. for 60 min; Fig. 9B). Except for the reduced level of stainable Fe$^{3+}$, microscopic examination of excised wild-type and opt3-2 embryos at torpedo (data not shown) and curled cotyledon stages of embryogenesis (Fig. 9, A and B) showed no obvious developmental defect in opt3-2 embryogenesis.

To determine if the reduced stored Fe in opt3-2 seeds affects seed germination and early seedling growth, wild-type and opt3-2 seeds were germinated on agar medium containing 0, 0.1, 5.0, and 50.0 μM Fe(III)-EDTA. We found no difference in germination rates of wild-type and opt3-2 seeds in all the Fe levels tested (data not shown). Seedlings derived from these seeds showed no difference in early growth, as measured by average root length and extent of shoot growth at 8 d after germination (data not shown). However, on Fe-starved plates, we found that opt3-2 seedlings had shorter roots than the wild type, and had smaller, bleached cotyledons compared with larger, light-green wild-type cotyledons (Fig. 9C). The comparable germination rates of wild-type and opt3-2 seeds in all the media tested indicated that Fe concentration in opt3-2 seeds, although reduced, was sufficient to supply Fe during the germination process. Moreover, the fact that opt3-2 plants attained wild-type-like seedling growth when exogenous Fe was provided indicates that opt3-2 seedlings are not compromised in their ability to acquire Fe from the growth medium. Notable differences between wild-type and opt3-2 plants were observed only during early seedling growth in the absence of exogenous Fe because Fe under this condition is derived solely from the seed.

We next examined the seed yield of opt3-2 compared with the wild type. We found that opt3-2 plants produced approximately 50% less seed yield than the wild type (Fig. 10A). To determine if the lower yield was due to less seed per silique produced by opt3-2, we determined silique length and seed number per silique of fully expanded wild-type and opt3-2 siliques (all at stage 17 of fruit development; Smyth et al., 1990; data not shown). We found that opt3-2 siliques (12.6 ± 1.1 mm, n = 139) were on average shorter than wild-type siliques (16.2 ± 0.7 mm, n = 109). We also found that opt3-2 plants contained on the average less seeds per silique (52 ± 4, n = 30) than the wild type (64 ± 2, n = 20). Representative wild-type and opt3-2 siliques and seeds are shown in Figure 10, B and C, respectively. As shown in Figure 10C, although seed yield was reduced in opt3-2, we observed no obvious difference in seed morphology (e.g. seed size, shape, and color) between opt3-2 and the wild type (Fig. 10C). Therefore, the reduction in seed yield is largely due to fewer siliques per plant.

In summary, the mobilization of Fe to seeds is compromised in opt3-2 plants, as indicated by reduced
Fe concentration and less stainable Fe$^{3+}$ in opt3-2 seeds compared with the wild type, as well as less growth and cotyledon greening in opt3-2 seedlings germinated on medium containing no exogenous Fe. Stored Fe in opt3-2 seeds, albeit decreased, is localized to the embryonic vasculature as found in the wild type. Moreover, except for reduced Fe concentration, no obvious defects in opt3-2 embryo and seed development were observed. In addition to compromised Fe mobilization to seeds, we also found that opt3-2 plants had shorter silique length, fewer seeds per silique and less seed yield per plant compared with the wild type.

**DISCUSSION**

**Role of AtOPT3 in Fe Homeostasis**

Excess Fe can be toxic to plants and hence its acquisition and distribution throughout the plant has to be tightly regulated. A very important and largely uncharacterized aspect of Fe homeostasis is the modulation of root Fe uptake activities in response to Fe availability. A model requiring at least two nutrient sensors, one for sensing the nutrient status of the shoot and one in the root that perceives the long-distance signal coming from the shoot, was proposed for systemic regulation of Fe responses in strategy I plants (Schmidt, 2003). The opt3-2 mutant exhibited Fe starvation phenotypes even when grown under Fe-sufficient conditions and, more importantly, even when in planta Fe levels were excessively high. Transport by AtOPT3, therefore, is important for shoot-to-root signaling that regulates Arabidopsis root Fe deficiency responses.

There are two possible signaling scenarios that would account for the constitutive root Fe-starvation phenotype of opt3-2. First, the opt3-2 shoot is compromised in sensing its Fe-replete status and, thus, constitutively generates an Fe deficiency signal to the root. Second, the opt3-2 shoot can sense its Fe status but the opt3-2 root constitutively perceives an Fe deficiency signal. The first scenario was proposed to account for the observed deregulation of root Fe deficiency responses in the pea (*Pisum sativum*) brz (bronze) and dgl (degenerative leaves) mutants (Grusak et al., 1990; Grusak and Pezeshgi, 1996). Reciprocal grafting experiments in these mutants showed that the aberrant root phenotype was indeed determined by the shoot, and not by the root, genotype (Grusak and Pezeshgi, 1996). Like opt3-2 leaves, the pea brz and dgl show, respectively, bronze necrotic spotted leaves and brown degenerative leaves due to hyperaccumulation of Fe. The genes corresponding to the pea brz and dgl mutations are still unknown and identifying such a component in Arabidopsis could be very useful for understanding the underlying signaling mechanism.

It is also possible that the opt3-2 is not a signaling mutant but, rather, is functionally Fe deficient. It is conceivable that Fe in opt3-2 shoots, although present in high amounts, may not be physiologically available, which then leads to generation of an Fe deficiency signal to roots. This was proposed for the tomato *chln* (Pich et al., 2001) and Arabidopsis *frd3* (Rogers and Guerinot, 2002; Green and Rogers, 2004) mutants. Both mutants exhibit obvious Fe deficiency symptoms (e.g. chlorosis and lethality) when exogenous Fe is not applied, and a lack of shoot ferritin in the case of *frd3*. Unlike these mutants, our results indicated that the opt3-2 shoot can accumulate sufficient intracellular Fe levels, i.e. opt3-2 shoots were not chlorotic and accumulated wild-type levels of chlorophyll and *FER1* transcripts. However, it is possible that only certain cell types in opt3-2 shoots are Fe deficient. For example, leaf palisade parenchyma cells in opt3-2 failed to differentiate, remaining rounded and indistinguishable from the underlying layer of spongy mesophyll cells. Palisade cells contain the largest per-cell number of chloroplast in plants and differentiation of prepalisade cells during leaf growth was proposed to depend on the developmental status of the chloroplast (Keddie et al., 1996). Undifferentiated palisade cells containing abnormal chloroplasts were observed in Arabidopsis Fe transporter mutants *irt1* and *pic1* (Henriques et al., 2002; Duy et al., 2007). It is possible that palisade parenchyma cell development is very sensitive to Fe availability and that whole-plant Fe status may be sensed, at least in part, by the Fe status of palisade cells.

High levels of Fe in the pea *brz* and *dgl* leaves resulted in the development of small necrotic spots, which enlarged with age until whole leaves became necrotic (Grusak et al., 1990; Welch and Larue, 1990; Grusak and Pezeshgi, 1996). Necrotic spots were also observed in...
opt3-2 leaves that became more pronounced with age and correlated with high levels of stainable \textit{Fe}^{3+}. High levels of stainable \textit{Fe}^{3+} were mostly localized in trichomes. Sequestration of heavy metals in trichomes appears to be a common means of metal detoxification in plants (Kupper et al., 2000; Choi et al., 2001; Sarret et al., 2006). Grayish spots were also observed in older opt3-2 silique, again in areas that coincided with high levels of stainable \textit{Fe}^{3+}. In addition to Fe, other metals such as Mn, Zn, and Cu were also overaccumulated in opt3-2 tissues. Accumulation of these micronutrients is likely not due to the opt3-2 mutation per se, but is a consequence of the deregulated metal transport in mutant roots via IRT1 (Welch et al., 1993; Cohen et al., 1998; Vert et al., 2002).

**Role of AtOPT3 in Seed Development**

Distribution of Fe in the plant requires long-distance transport in the vascular tissue. Once taken up by the root, Fe in the xylem is translocated into the aerial parts through the transpiration stream. Because the xylem sap moves up the plant through transpiration, xylem mineral transport to nonexposed reproductive tissues (seeds and fruits) and developing leaves and roots is low to nonexistent (Grusak, 2002). Mineral transport, including Fe, to these tissues, is believed to occur exclusively via the phloem transport system (Hocking and Pate, 1977, 1978; Grusak, 1994; Zhang et al., 1995). We previously hypothesized that AtOPT3 is likely involved in the mobilization of Fe to developing seeds (Stacey et al., 2006). The data we present in this article further support this hypothesis. First, mobilization of Fe to seeds is compromised in opt3-2, as indicated by reduced Fe concentration and less stainable \textit{Fe}^{3+} in opt3-2 seeds compared with the wild type, as well as less growth and cotyledon greening in opt3-2 seedlings germinated on medium containing no exogenous Fe. Second, although Fe concentration in opt3-2 seeds is reduced, overaccumulation of Fe and other micronutrients was observed in all other opt3-2 tissues analyzed. One can argue that the decreased Fe concentration in opt3-2 seeds could be due to other defects in seed development not directly related to Fe transport. However, the lack of any obvious developmental defects in opt3-2 seeds, the Fe-regulated expression of AtOPT3, and the various defects in Fe metabolism exhibited by opt3-2 all argue for a critical role for AtOPT3 in Fe mobilization rather than a role in some unknown developmental process(es) during seed development. AtOPT3 is essential for leaf palisade parenchyma cell development, likely in the transport of Fe required for normal chloroplast development. It is also likely that the embryo-lethal phenotype of the opt3-1 knock-out mutant (Stacey et al., 2002b) is due to compromised Fe

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**Figure 9.** Reduced stainable \textit{Fe}^{3+} in developing opt3-2 seeds and reduced growth of opt3-2 seedlings under Fe-starved conditions. A, Localization of \textit{Fe}^{3+} in developing embryos of Col-0 and opt3-2 seeds at early (excised embryos, top) and late (bottom) curled-cotyledon stages of embryo development. Arrows indicate stored Fe in axis and cotyledons of Col-0 embryo. Perl’s staining to visualize \textit{Fe}^{3+} was done for 30 min. Arrows indicate \textit{Fe}^{3+} in embryonic vasculature. Bars = 120 \textmu m. B, Localization of \textit{Fe}^{3+} in excised opt3-2 embryos after 30 sec (left and middle) and 60 (right) min of Perl’s staining. Similar to Col-0, longer staining period showed that \textit{Fe}^{3+} was localized to developing vasculature of opt3-2 embryos. Arrows indicate \textit{Fe}^{3+} in embryonic vasculature. Bars = 120 \textmu m. C, Seedling growth of Col-0 and opt3-2 under Fe-starved condition. Seeds were germinated and grown horizontally for 8 d on medium lacking Fe. Bars = 5 mm.
Representative fully extended siliques from Col-0 and seeds. Bars 5 mm. A, Representative green and desiccated Col-0 and opt3-2 plants are shown. B, opt3-2 at 10 d after bolting. C, Representative green and desiccated Col-0 and opt3-2 seeds. Bars = 500 μm (top) and 300 μm (bottom).

Figure 10. Reduced seed yield in opt3-2. A, Seed yield per plant. Means and sd obtained for 10 Col-0 or 10 opt3-2 plants are shown. Representative fully extended siliques from Col-0 and opt3-2 plants are shown. Siliques were obtained from approximately similar positions on the main inflorescence stems of Col-0 and opt3-2 at 10 d after bolting. Bar = 4 mm. C, Representative green and desiccated Col-0 and opt3-2 seeds. Bars = 500 μm (top) and 300 μm (bottom).

transport to seeds and, subsequently, to abnormal plastid development in developing embryos. There are several reports implicating the essentiality of plastid development in embryogenesis (Uwer et al., 1998; Bellaoui et al., 2003; Ma and Dooner, 2004). So far, little is known about the transport processes involved in the mobilization of Fe from source to sink tissues. These processes would include movement and loading of Fe in the phloem, long-distance phloem transport, and subsequent phloem unloading and post-phloem transport into sink tissues. Transported Fe can come from remobilized stored Fe in source tissues and from Fe derived from concurrent xylem transport from the root (Grusak, 1994). The overaccumulation of Fe in source tissues of opt3-2 indicate that AtOPT3 could be involved in the movement of Fe into the phloem transport system in these tissues. For example, overaccumulation of Fe in opt3-2 rosette leaves became more pronounced during the seed-filling stage (i.e. at 10 d after bolting) and most of the accumulated Fe in opt3-2 siliques did not get into seeds but remained in the seed pods. Other OPT transporters were proposed to function in processes involved in the mobilization of Fe to developing seeds. These include the rice OsYSL2 (Koike et al., 2004) and the Arabidopsis AtYSL1 and AtYSL3 (Le Jean et al., 2005; Waters et al., 2006). OsYSL2 is expressed in phloem companion cells and was suggested to be involved in the phloem transport of Fe and Mn into the grain. Like AtOPT3, OsYSL2 expression is up-regulated by Fe deficiency (Koike et al., 2004; Stacey et al., 2006).

AtOPT3 could also function in the post-phloem movement of Fe to developing seeds. This function is consistent with the expression of AtOPT3 in seeds immediately following fertilization and throughout seed development (Stacey et al., 2002b). There are three apoplastic borders between the phloem and the embryo where carrier-mediated transport seems necessary. These include apoplastic borders between the outer and inner integument, between the inner integument and the endosperm, and between the endosperm and the embryo (Stadler et al., 2005). AtOPT3 is expressed in integuments, endosperm, and embryo and could be involved in the post-phloem transport of Fe across the apoplastic borders between these tissues (Stacey et al., 2002b). It was suggested that the suspensor has a nutritive function in young embryos (Yeung and Meinke, 1993). Expression of AtOPT3 in suspensor cells would also indicate a possible role for AtOPT3 in Fe uptake via the suspensor. Lastly, AtOPT3 is expressed predominantly in prevascular tissues of developing embryos, the sites where stored Fe is accumulated in Arabidopsis seeds (Kim et al., 2006).

Our data clearly indicate that AtOPT3 function is crucial for the long-distance transport of Fe to developing seeds. However, further studies are needed to elucidate the specific transport process(es) that is mediated by AtOPT3. The fact that opt3-2 plants were not chlorotic and overaccumulated Fe in their shoots indicates that the mutant is not compromised in the root-to-shoot transport of Fe. It is therefore unlikely that AtOPT3 is critical for Fe mobilization through the xylem transport system. However, because opt3-2 is not a null mutant, we cannot rule out the possibility that AtOPT3 has a function in xylem transport of Fe as well. The nature of the substrate transported by AtOPT3 remains to be determined.

In conclusion, we propose that AtOPT3 mediates the transport of a peptide/modifed peptide Fe chelator or Fe-chelator complex that is critical for Fe nutrition of the embryo and for long-distance signaling of whole-plant Fe status. Moreover, AtOPT3 functions in the transport of this complex to seeds, via the phloem transport system, and to cells or organs that are involved in shoot-to-root signaling of Fe deficiency responses.

MATERIALS AND METHODS

Isolation of the opt3-2 Allele, Genetic Analysis, and Complementation

The mutant line SALK_021168 was identified from the SALK (Salk Institute) collection of T-DNA insertional mutants (Alonso et al., 2003). Isolation of homozygous mutant plants was done by PCR-based screening using a T-DNA left border-specific primer, 5'-GGTAGTGCTACCAGTGGCCCAT-3', and the AtOPT3-specific primers 5'-AGAAGTGTTGGAGAGAATGAGAAGATG-3' and 5'-AATCTCCACTCTGAAATATCCGCTTA-3'. Insertion of the T-DNA upstream of the AtOPT3 gene was confirmed by sequencing the PCR product using the T-DNA-specific primer 5'-GGAACCACTGGACCCCTGGCAGAC-3'. Sequencing results showed that the T-DNA is inserted 36 bp upstream of the AtOPT3 start codon. The mutant line, designated opt3-2, showed a seed-lethal phenotype comparable to that previously reported for the N4 opt3 mutant (opt3-1). Co-segregation analysis of the embryo-lethal phenotype, however, showed that it is not due to opt3-2 but to a second-site mutation (data not shown). Homozygous opt3-2 plants showing no seed lethality were then backcrossed to Col-0. Twenty-eight F2 plants derived from the cross were genotyped by PCR to isolate opt3-2 homozygotes (data not shown). For genetic complementation of the opt3-2 mutation, mutant plants were transformed with an AIOPT3 genomic DNA expressed from the AIOPT3 promoter (Stacey et al., 2002b). As control, opt3-2 plants were also transformed with an AIOPT3 promoter-GUS construct (Stacey et al., 2002b). Transgenic plants were selected for hygromycin resistance. For seed amplification, opt3-2 and transgenic plants were allowed to self-fertilize.

Plant Growth Conditions and Transformation

Routine seedling growth was done aseptically on agar medium containing half-strength Murashige and Skoog salts (Sigma) and 1% Suc (w/v) supple-

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mented as required with 25 μg/mL hygromycin. For Fe(II)-chelate reductase activity assays, RNA isolations, and chlorophyll determinations, plants were grown on half-strength Murashige and Skoog medium for 15 d, transferred to Fe-sufficient or Fe-deficient medium (Yi and Guerinot, 1996), and grown for an additional 4 d. For seed amplification and analysis of mature plants, 10-d-old seedlings were transferred to Pro-Mix soil (Pretset Horticulture) and grown at 22°C under 120 μmol m⁻² s⁻¹ of constant fluorescent white light. Stable transformation of Arabidopsis (Arabidopsis thaliana) was performed following the vacuum infiltration procedure for Agrobacterium tumefaciens-mediated T-DNA gene transfer (Bechtold and Pelletier, 1998).

**Fe(III)-Chelate Reductase Assays and Determination of Chlorophyll Content**

For root Fe(III)-chelate reductase activity determinations, seedlings were placed in 1 mL of solution containing 300 μM ferrozine (Sigma) and 100 μM Fe(III)-EDTA. The enzymatic reaction was allowed to proceed for 60 min, after which A₄₅₀ was determined. Roots were detached and weighed. For chlorophyll content determinations, shoot tissues were detached and chlorophyll was extracted in methanol. Chlorophyll concentration was calculated as previously (Porra et al., 1989) using the A₅₃₂, A₆₅₂, and A₆₆₅ obtained for each extraction.

**Perl’s Staining for Fe³⁺, Tissue Sectioning, and Microscopy**

For localization of Fe³⁺, Arabidopsis seedlings or tissues were vacuum infiltrated with Perl’s stain solution (equal volumes of 4% [v/v] HCl and 4% [v/v] K-ferricyanide) for 15 min. Plant samples were incubated for another 15 min in the stain solution and were rinsed three times with water. Localization of Fe³⁺ was observed and imaged using a Nikon SMZ 1500 microscope equipped with Nikon DXM 1200 digital camera.

For developmental examination of leaf cells, leaves from adult plants were obtained at bolting and were fixed overnight at 4°C in 50 mM phosphate buffer (pH 7.2) containing 1% glutaraldehyde and 4% paraformaldehyde. Fixed tissues were dehydrated in 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, and 90% ethanol for 15 min at each concentration, then in 100% ethanol for 1 h. Tissues were embedded in JB-4 embedding kit (Electron Microscopy Science) following the manufacturer’s protocol. Transverse sections (2.5 μm) were obtained using a Leica RM 2065 microtome. Sections were stained with 0.05% toluidine blue, mounted in Eukitt (O. Kindler GmbH) and observed using a Nikon Alphaphot-2 microscope equipped with a Nikon CoolPIX 885 camera.

**Determinant of Macro- and Micronutrient Concentration**

Wild-type and opt3-2 tissues were analyzed at two stages of reproductive growth: (1) bolting stage, when the primary inflorescence was approximately 0.5 to 2 cm in length; and (2) 10 d after bolting when few flower buds remained. At bolting, aerial parts of 12 plants were harvested, pooled, and analyzed. At 10 d after bolting, rosette and the oldest siliques were not yet dehiscent. At bolting, aerial parts of 12 plants were harvested, pooled, and analyzed. Nutrient concentration in harvested wild-type, inflorescence stems, and siliques from 12 plants were harvested, pooled, and analyzed. Fe(III)-chelate reductase activity determinations, DNA cloning, Perl’s staining, and plant genotyping procedures. We also thank Karl-Henrik K. Lindell and Aaron J. Witte for their assistance in growing plants and obtaining plant materials used in this study. We are also grateful to Dr. Michael Grusak for his helpful comments on the data that were presented in this manuscript.

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