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ORIGINAL ARTICLE

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Expression analyses of *Arabidopsis* oligopeptide transporters during seed germination, vegetative growth and reproduction

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Abstract *AtOPT* promoter-GUS fusions were constructed for six of the nine known, putative oligopeptide transporters (OPTs) in *Arabidopsis thaliana* and used to examine *AtOPT* expression at various stages of plant development. *AtOPT1*, *AtOPT3*, *AtOPT4*, *AtOPT6* and *AtOPT7* were expressed in the embryonic cotyledons prior to root radicle emergence. Except for *AtOPT8*, which gave weak expression, all *AtOPT*s were strongly expressed in post-germinative seedlings with strongest expression in vascular tissues of cotyledons and hypocotyls. Preferential expression of *AtOPT*s in vascular tissues was also observed in cotyledons, leaves, hypocotyls, roots, flowers, siliques, and seed funiculi of seedlings and adult plants. Differential tissue-specific expression was observed for specific *AtOPT*s. For example, *AtOPT1*, *AtOPT3* and *AtOPT8* were uniquely expressed in pollen. Only *AtOPT1* was expressed in growing pollen tubes, while only *AtOPT6* was observed in ovules. *AtOPT8* was transiently expressed in seeds during early stages of embryogenesis. Iron limitation was found to enhance expression of *AtOPT3*. These data suggest distinct cellular roles for specific AtOPTs including nitrogen mobilization during germination and senescence, pollen tube growth, pollen and ovule development, seed formation and metal transport.

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Introduction

Peptide transport is characterized by the ability of cells to transport peptides or peptide derivatives across membranes in an energy-dependent manner. Peptide transporters can be classified into three groups, namely the ATP-binding cassette transporters (ABC family), the peptide transporters (PTR family) and the oligopeptide transporters (OPT family). Plant ABC transporters use ATP hydrolysis to energize transport and include the *Arabidopsis* MRPs (multidrug-resistance-associated proteins), which were shown to transport glutathione (γ-Glu-Cys-Gly), glutathione S-conjugates and glucoronides (reviewed by Blackmore et al. 2001; Detmers et al. 2001; Rea 1998). PTR transporters use the proton gradient to drive transport. PTR proteins were shown to transport a wide variety of nitrogen-containing substrates such as amino acids (Frommer et al. 1994; Zhou et al. 1998), short peptides (di- and tripeptides) (Song et al. 1996; Steiner et al. 1994; Chiang et al. 2004) and nitrate (Tsay et. al. 1993). Although PTR proteins are well characterized from bacteria, fungi and human, plant PTRs are less studied (reviewed by Stacey et al. 2002a; Williams and Miller 2001). Sequence comparisons identified 51 putative *PTR* genes in the *Arabidopsis* genome (Stacey et al. 2002a).

Members of the OPT family were first characterized in yeast and likely use the proton gradient to energize transport (Lubkowitz et al. 1997, 1998). In contrast to PTR peptide transporters which recognize di- and tripeptides, the yeast OPT transporters predominantly recognize tetra- and pentapeptides ((Lubkowitz et al. 1997; Lubkowitz et al. 1998), including the endogenous opioids Met-enkephalin (YGGFM) and Leu-enkephalin (YGGFL) (Hauser et al. 2000). The *Saccharomyces cerevisiae* Opt1p was also shown to be a high-affinity

glutathione transporter (Bourbouloux et al. 2000). Database sequence comparisons with *Candida albicans* CaOpt1p identified nine putative *Arabidopsis* OPT transporters, AtOPT1 to AtOPT9 (Koh et al. 2002). These AtOPTs share 49–53% sequence similarity to CaOPT1p and show no sequence similarity to ABC or PTR transporters. OPT proteins were also identified in other plants, namely, the *Brassica juncea* BjGT1 (Bogs et al. 2003) and the *Oryza sativa* OsGT1 (Zhang et al. 2004). The plant OPTs form a distinct subfamily compared to the fungal OPTs (Yen et al. 2001; Koh et al. 2002; Zhang et al. 2004).

Koh et al (2002) showed that expression of AtOPTs 1, 4, 5, 6 and 7 (of 7 AtOPTs tested) in yeast enabled the uptake of at least one of the synthetic peptides KLLG, KLGL or KLLLG (Koh et al. 2002). More recently, BjGT1 (Bogs et al. 2003), OsGT1 (Zhang et al. 2004), and AtOPT6 (Cagnac et al. 2004) have been reported to enable the uptake of glutathione (GSH and GSSG) and glutathione derivatives (GS conjugates) when expressed in yeast. Subsequent to the identification of the first plant OPT transporters, more extensive sequence comparisons led to the identification of a more extended protein family (Yen et al. 2001). These include the *Myxococcus xanthus* EspB protein (Cho and Zusman 1999), involved in temporal pattern development during sporulation, and the *Zea mays* Yellow Stripe 1 (YS1) protein, shown to transport Fe^{3+}-phytosiderophore (Curie et al. 2001) and Fe^{2+}-nicotianamine (NA; Roberts et al. 2004; Schaaf et al. 2004) complexes. There are eight full-length *Yellow Stripe-Like* (*AtYSL*s) genes in the *Arabidopsis* genome (DiDonato et al. 2004), forming a cluster phylogenetically distinct from the AtOPTs. The OPT family of transporters, therefore, is larger and more diverse than was previously recognized. However, no OPT transporter has yet been identified in animals. Although OPT proteins were originally characterized as tetra- and pentapeptide transporters, it now appears that OPT transporters can transport more diverse substrates, all of which are peptide-containing compounds or peptide derivatives.

We previously reported on the identification and characterization of a loss of function mutation in the *Arabidopsis AtOPT3* gene (Stacey et al. 2002b). We found that AtOPT3 was crucial for normal embryogenesis, as indicated by the embryo-lethal phenotype of plants homozygous for the *opt3* mutant allele. Mutant embryos were arrested very early in development, mostly at the two- to eight-cell stage of embryogenesis. Consistent with the embryo-lethal phenotype associated with *opt3*, *AtOPT3* expression was localized in the endosperm tissues, integuments and, most notably, in the embryos of developing seeds. To date, only two plant OPT members have been reported to show a mutant phenotype, *AtOPT3* and the maize *ZmYS1*, where mutants exhibited a chlorotic phenotype due to a defect in iron uptake (von Wiren et al. 1994; Curie et al. 2001). The lack of observable phenotypes in loss-of-function mutants of other *AtOPT*s may be the result of functional redundancy among the family members. Knowledge of the expression pattern of individual genes can be helpful in designing multiple knock-out lines, and also in elucidating physiological function. Koh et al. (2002) previously reported on the tissue-specific expression of seven of the nine *AtOPT*s (*AtOPT1* to *AtOPT7*) by semi-quantitative RT-PCR. We now expand on this effort by analyzing *AtOPT* promoter-GUS fusion expression in transgenic *Arabidopsis*. The results showed that the *Arabidopsis AtOPT*s (*AtOPT*s *1*, *4*, *6*, *7* and *8*) gave comparable expression to that previously reported for *AtOPT3* (Stacey et al. 2002). All were preferentially expressed in the vascular tissues of vegetative and reproductive tissues, and none was expressed in root hairs and root tips. Additional unique expression patterns were also found for selected *AtOPT*s. For example, *AtOPT1* was expressed in pollen tubes, *AtOPT6* was expressed in ovules and *AtOPT8* was expressed in the endosperm and integument of developing seeds. Iron limitation was found to uniquely enhance the expression of *AtOPT3*.

Materials and methods

Plant growth conditions and transformation

Seeds were surface-sterilized with a 35% bleach solution containing 0.1% Triton X-100 for 10 min, rinsed several times with sterile water, and cold-treated for 2–3 days. For routine growth, seedlings were grown on agar medium containing one-half-strength Murashige and Skoog (MS) salts (Sigma, St. Louis, MO, USA), MS vitamins (Sigma), 1% sucrose (w/v), 0.05% MES, and 25 μg/ml hygromycin. For seed amplification and analysis of mature plants, 10- to 15-day-old seedlings were transferred and grown on Premier Pro-Mix soil (Quakertown, PA, USA). Plants were grown at 22°C under constant fluorescent white light.

For growth in iron-sufficient and iron-limiting conditions, surface-sterilized, cold-treated seeds were germinated on agar medium containing Gamborg's B-5 salts (Sigma), 1% sucrose (w/v), 0.05% MES, and 25 μg/ml hygromycin. Seedlings were grown until the four- to six-true leaf stage (12–14 days) and transferred either to agar medium containing salts as described by Masrchner et. al. (1982), 0.05% MES and 50 μM Fe(III) EDTA for iron-sufficient plates or 300 μM ferrozine (Sigma) for iron-deficient plates. Seedlings were analyzed after 5 days of growth in iron-sufficient or iron-deficient conditions. All seedlings were grown under yellow 0.118 in. plexiglass (Delvie's Plastics, Inc, Salt Lake City, UT, USA) at 22°C and constant fluorescent white light. Stable transformation of *Arabidopsis* (ecotype Columbia, Col-O) was done following the vacuum infiltration procedure for *Agrobacterium tumefaciens*-mediated T-DNA gene transfer (Bechtold and Pelletier 1998).

Cloning of AtOPT promoter–GUS fusions

AtOPT promoter sequences were amplified from *Arabidopsis thaliana* ecotype Col-O genomic DNA and cloned upstream of the promoterless *β*-glucoronidase (*GUS*) gene encoded in the binary vector pCambia1391Z (Hajdukiewicz et al. 1994). Primers and promoter fragment size used for cloning each *AtOPT* promoter are as follows: *AtOPT1*, 5′-GAGATCTCGATCTTGTTGGTACAAGCGTGCT-3′ (forward primer) and 5′-GCCTAGGTGTCTTCAAGCTCCTTTTTTCCGT-3′, 3.2 kbp; *AtOPT4*, 5′-GGTCGACTGTGTTTCTGGAGAGCCATTGTAA-3′ (forward primer) and 5′-GCCTAGGTCCGGTTTAGTTTGTGTGTGAGTG-3′ (reverse primer), 2.1 kbp; *AtOPT6*, 5′-CTCAAGCTTAAAGGAGGAGCCGATGAAGTGATATTCTT-3′ (forward primer) and 5′-CTGCCTAGGGTTTTAGAAGAGTGAGTGTTCCGTTCTTT-3′ (reverse primer), 2.2 kbp; *AtOPT7*, 5′-CAGGATCCTGTATTAGCACTAACGAAATCTTT-3′ and 5′-CAGAATTCTTTGGGGAGATGTGAATCTGACAA-3′ (reverse primer), 2.2 kbp; *AtOPT8*, 5′-GAGATCTGCATACTACATTCTCCCTGGTTAT-3′ (forward primer) and 5′-GCCTAGGCGGTCTTCAGAAACAGAGGATTGG-3′ (reverse primer), 3.4 kbp. Cloning of the *AtOPT3* promoter-GUS fusion was previously described (Stacey et al., 2002). Promoter fusions for *AtOPT2* (3.5 kbp), *AtOPT5* (3.4 kbp) and *AtOPT9* (3.0 kbp) were also cloned but gave no or very weak GUS activity *in planta*.

Histochemical GUS staining and microscopy

Transgenic *Arabidopsis* plants expressing *AtOPT* promoter–GUS fusions were stained for GUS using 5-bromo-4-chloro-3-indoyl-*β*-D-glucoronide (X-Gluc; Gold Biotechnology, Inc., St. Louis, MO, USA) as substrate according to published protocols (Jefferson et al. 1987). Stained seedlings and tissues were fixed in FAA solution (50% ethanol, 5% acetic acid, 3.7% formaldehyde) and destained in 70% ethanol overnight. Additional overnight clearing with lactophenol (1 H_2O:1 glycerol:1 lactate:2 phenol, v/v) was done for unfertilized gynoecia and young siliques to visualize internal parts such as ovules, pollen tubes and developing seeds. In certain cases, seeds were dissected from the ovary and cleared with lactophenol to visualize the embryos. Staining patterns were observed and documented using a Nikon microscope (SMZ1500) equipped with a Nikon digital camera (DXM1200). For each *AtOPT* promoter-GUS fusion, at least 25 independent T1 lines were examined for GUS activity in leaves. Transgenic lines expressing a GUS fusion to *AtOPT1*, *AtOPT4*, *AtOPT6*, *AtOPT7* or *AtOPT8* gave similar leaf-expression patterns except for differences in expression levels, which ranged from very weak to strong. Three to four lines showing strong expression were selected for each *AtOPT* promoter fusion and corresponding T2 progenies were analyzed at various stages of development, namely, during germination, at the two–four seedling stage, and adult plants within 1 week of bolting. The expression of each *AtOPT*–GUS fusion was also analyzed at various stages of flower development as described by Smyth et al. (1990) and, subsequently, in developing seeds. Transgenic lines expressing a GUS fusion to *AtOPT2*, *AtOPT5* or *AtOPT9* showed little to no detectable GUS staining in leaves of T1 plants and were not analyzed further.

Quantitative GUS activity assays

GUS activity was measured fluorometrically for intact tissues using 4-methylumbellyferyl-*β*-D-glucoronide (4-MUG; Gold Biotechnology, Inc., St Louis, MO, USA) as substrate, as described by Jefferson et al. (1987). Fluorescence was measured in a spectrofluorometer equipped with a microplate reader. For each *AtOPT* promoter–GUS fusion, at least three independent lines were analyzed. Fluorometric assays were done in three trials, with two replicates per trial. Four to five transgenic seedlings were assayed per replicate. GUS activity for each sample was normalized per unit seedling wet weight.

Cloning of *AtOPT3* cDNA and transport assays in *S. cerevisiae* and *Xenopus* oocytes

To clone the *AtOPT3* cDNA, total RNA isolated from Col-0 plants was used as a template for reverse-transcriptase-based (RT) PCR using random hexamer primers. First-strand cDNA was amplified with Pfu DNA polymerase (Stratagene, La Jolla, CA, USA) using primers 5′-GGCCAACAACGCAACTTTGTCTGGTACTTCA-3′ (forward primer) and 5′-CTGCTGAGCTTAGAAAACGGGACAGCC-3′ (reverse primer). Amplified PCR products were cloned into pGEM T Easy cloning vector (Promega, Madison, WI, USA) and sequenced. For functional expression in *S. cerevisiae* or *Xenopus* oocytes, the full-length *AtOPT3* cDNA clone was digested with *Not*I and subcloned into the yeast expression vector pDB20 (Becker et al. 1991) or the oocytes expression vector pGEMHE (Liman et al. 1992), respectively. An artificial *Not* I site in pGEMHE was created by swapping *Eco*RI–*Xba*I sites with *Eco*RI- and *Xba*I-digested multicloning sites of pCR 2.1 TOPO cloning vector (Invitrogen, Carlsbad, CA, USA). Transformation of *S. cerevisiae* BY4730 (MATα *leuΔ0 met15Δ0 ura3Δ0*) was done following the LiOAc method. Transformed cells were selected by growth on 0.17% (w/v) YNB plates supplemented with 2% (w/v) glucose lacking uracil. Yeast growth assays in the presence of 100 μM KLGL, KLLLG, YGGFL or GSH were performed according to previously described protocol (Koh et al. 2002). AtOPT3 and ScOPT1 (Hauser et al. 2000) expression in *Xenopus* oocytes was performed as previously described (Rubio et al. 1995). Oocytes, 4 days

after injection with 23 ng of *AtOPT3* or *ScOPT1* cRNA, were voltage-clamped in a bath solution containing 1 mM KCl, 6 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM Mes–tris (pH 5.0), and 1 mM GSH. Osmolality of the bath solution was adjusted to 240 mosmol kg^{-1} by the addition of D-mannitol.

Results

Expression of *Arabidopsis OPT*s during germination and early seedling growth

To examine the possible role of *AtOPT*s in storage protein mobilization during germination, we analyzed *AtOPT* promoter–GUS fusion activity in germinating seeds prior to radicle protrusion (germination stage) and in newly germinated seedlings (post-germination stage). *AtOPT1* was expressed at a low but detectable level in the cotyledons of germinating seedlings (Fig.1a, b), as well as in the cotyledons and prevascular tissues of newly germinated seedlings (Fig. 1c). Similar expression patterns were observed for *AtOPT3* and *AtOPT4* at the germination (data not shown) and post-germination stages. However, stronger *AtOPT3* expression was observed in the prevascular bundles of hypocotyls of postgerminative seedlings (Fig. 1d) when compared to *AtOPT4* (Fig. 1e). Likewise, *AtOPT6* (Fig. 1f, g) and *AtOPT7* (Fig. 1i) were also expressed in cotyledons of germinating seeds but at a much higher level than *AtOPT1*. Relatively higher expression levels of *AtOPT6* (Fig. 1h) and *AtOPT7* (Fig. 1j) were also observed in the cotyledons and prevascular tissues of hypocotyls of postgerminative seedlings. In contrast, *AtOPT8* was poorly expressed in germinating seeds and seedlings (Fig. 1k), showing detectable GUS staining only after prolonged staining (Fig. 1l). For all *AtOPT* tested, no detectable expression was observed in young roots or root hairs (Fig. 1c–e, h, j–l). In summary, with the exception of *AtOPT8*, all the *AtOPT*s examined showed significant expression in cotyledons of pregerminative seeds and newly germinated seedlings. Moreover, although differences in expression levels were observed among the various *AtOPT*s in postgerminative seedlings, similar expression patterns were observed, namely, predominant expression in prevascular tissues of cotyledons and hypocotyls and lack of expression in radicles and root hairs.

Expression of *Arabidopsis OPT*s during vegetative growth

In young seedlings (at the two to four true-leaf stage), strong *AtOPT1* expression was observed in the vascular tissues of cotyledons (Fig. 2a), hypocotyls (Fig. 2b) rosette leaves (Fig. 2a, c) and roots (Fig. 2d). *AtOPT1* was also expressed in trichomes (Fig. 2c), but no detectable expression was observed in root hairs (Fig. 2d) and in root tips (Fig. 2e). Similar expression patterns were observed in cotyledons, rosette leaves and hypocotyl for *AtOPT4* (data not shown), *AtOPT6* (data not shown) and *AtOPT7* (Fig. 2f), although the latter was expressed in all cells of the hypocotyl (Fig. 2g). *AtOPT8* was also expressed strongly in vascular tissues of cotyledons (Fig. 2h) and hypocotyl (Fig. 2i). However, unlike the other *AtOPT*s, *AtOPT8* was predominantly expressed in primary veins of rosette leaves (Fig. 2h). Comparable root expression to that of *AtOPT1* was observed for *AtOPT4*, *AtOPT6*, *AtOPT7* and *AtOPT8*, namely, high expression in the vascular cylinder but no detectable expression in root hairs and root tips (data not shown). *AtOPT6* expression was observed in emerging lateral roots for AtOPT6 but not for the other AtOPTs (data not shown).

In adult plants, all the *AtOPT*s showed predominant expression in vascular tissues of cauline (data not shown) and rosette leaves, as well as rosette leaf petioles. Representative GUS staining patterns in rosette leaves and leaf petioles expressing *AtOPT8* promoter–GUS are shown in Fig. 2j, and k, respectively. In inflorescence stems, very weak expression was detected for *AtOPT1* (Fig. 2l) and *AtOPT8* (data not shown), whereas strong expression, predominantly in vascular tissues, was observed for *AtOPT4* (Fig. 2m, n), *AtOPT6* (data not shown) and *AtOPT7* (Fig. 2o). Expression of *AtOPT3* in inflorescence stems was not examined.

Expression of *Arabidopsis OPT*s during flower development and fertilization

No detectable *AtOPT1* expression was observed in immature flowers (up to stage 11 of flower development; Fig. 3a). Also, no detectable expression was observed for *AtOPT3*, *AtOPT4*, *AtOPT6*, *AtOPT7* and *AtOPT8* during early stages of flower development (data not shown). At stage 12, detectable expression of *AtOPT1* was observed in sepals (Fig. 3b), predominantly in vascular tissues, and subsequently in anthers and pollen grains (Fig. 3c, d). We previously reported that *AtOPT3* was expressed in pollen grains (Stacey et al. 2002). Further examination of *AtOPT3* expression showed that it was expressed in pollen grains and anthers starting at stage 12 of flower development (data not shown). *AtOPT1* expression in stigma and in vascular tissues of the style (Fig. 3e) and petals (data not shown) was observed in only a portion (approximately 50%) of flowers examined. No expression in ovules or any part of the ovary was observed prior to anthesis (Fig. 3e). However, immediately following anthesis, *AtOPT1* was expressed along the central septum of the ovary (Fig. 3f).

AtOPT4 (Fig. 3g, h) and *AtOPT7* (data not shown) gave comparable expression patterns in unfertilized and fertilized flowers. Both showed relatively lower expression in flowers when compared to *AtOPT1*, notably weaker expression in sepals (Fig. 3g) and no detectable expression in pollen grains, anthers and ovary (Fig. 3g,

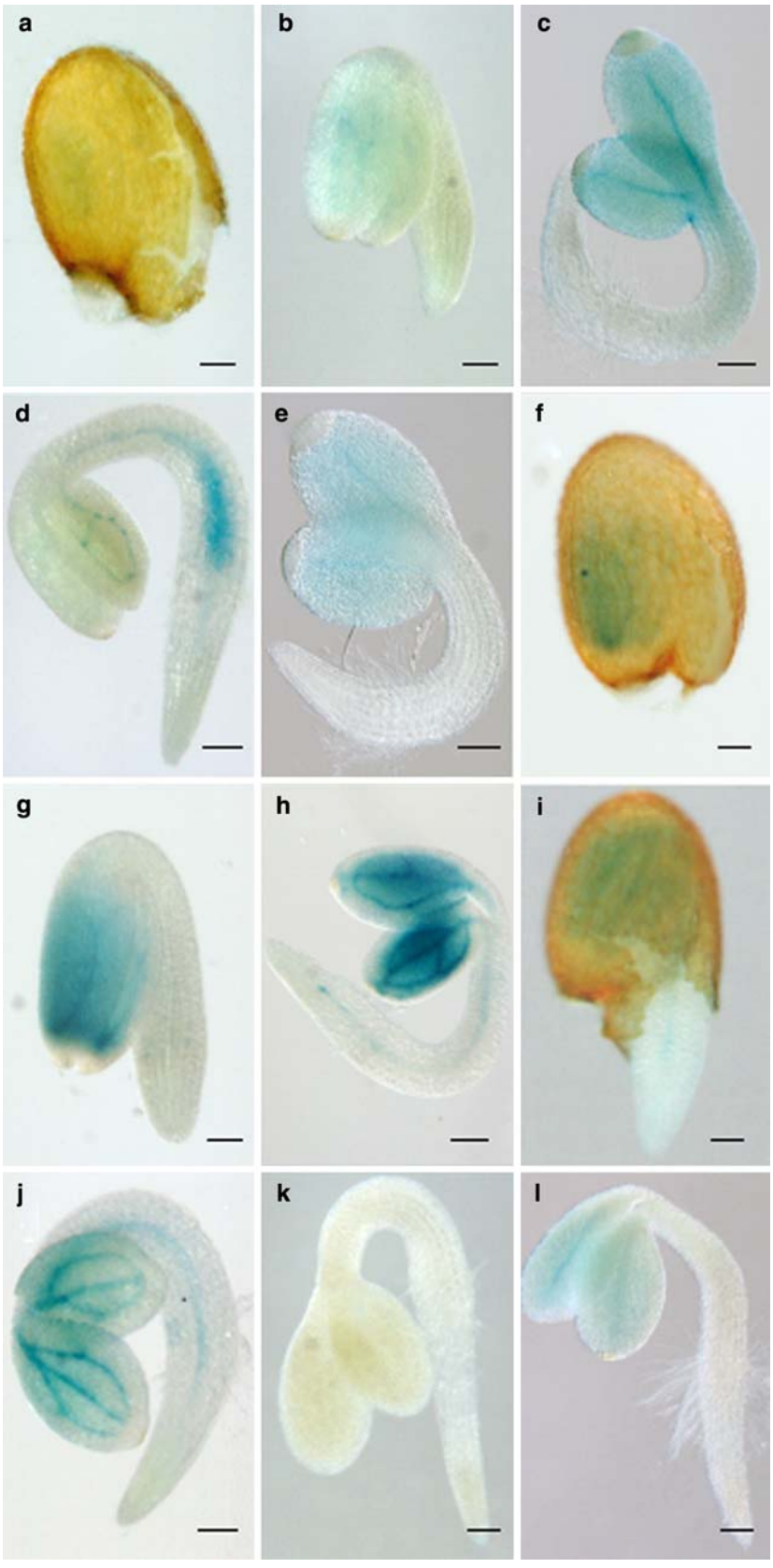

Fig. 1 a-l Histochemical localization of *AtOPT* promoter–GUS expression during *Arabidopsis* seed germination (**a**, **b**, **f**) and post-germinative seedling growth (**c–e**, **g**, **h–l**) Representative GUS staining patterns of transgenic *Arabidopsis* expressing *AtOPT1* (**a–c**), *AtOPT3* (**d**), *AtOPT4* (**e**), *AtOPT6* (**f–h**), *AtOPT7* (**i**, **j**) and *AtOPT8* (**k**, **l**) fusions are shown. *Bars* = 60 μm (**a**, **b**, **f**, **g**, **i**), 100 μm (**c**, **d**, **e**, **h**, **j–l**)

h). Like *AtOPT1*, *AtOPT4* and *AtOPT7* were expressed in stigma and vascular tissues of style in only a portion of flowers examined. This lack of detectable expression in a portion of flowers could be due to lower expression levels in this subset of flowers and/or possibly to less permeation of the substrate during staining.

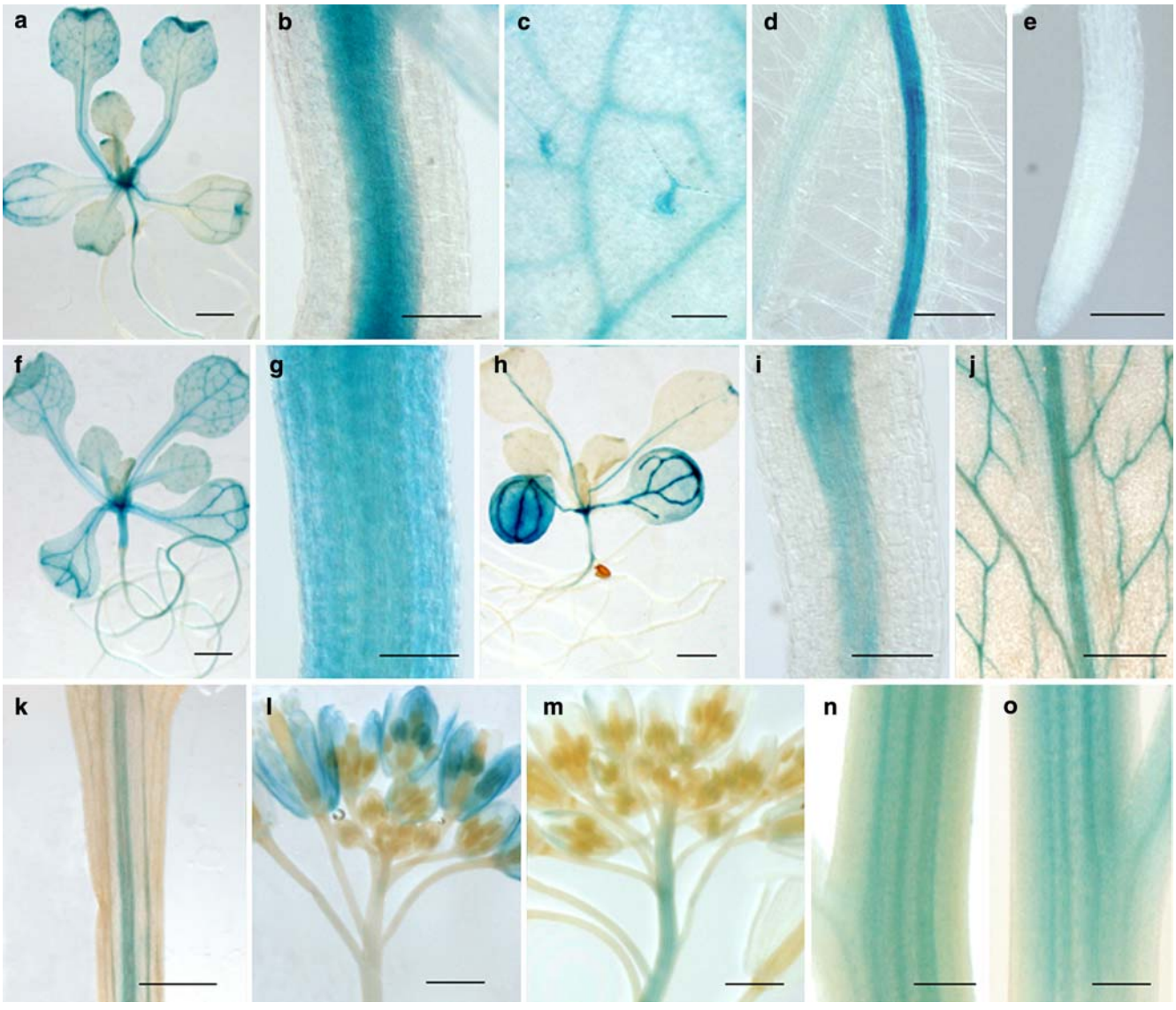

Fig. 2 a–o Histochemical localization of *AtOPT* promoter-GUS expression during vegetative growth and in inflorescence stems of transgenic Arabidopsis. **a–e** Representative expression patterns of *AtOPT1* expression in whole seedling (**a**), hypocotyl (**b**), trichomes and vascular tissues of rosette leaf (**c**), root (**d**), and root tip (**e**). **f, g** Representative expression patterns of *AtOPT7* expression in whole seedling (**f**) and hypocotyl (**g**). Note darker GUS staining in older leaves in (**a**) and (**f**). **h**, **i** Representative expression patterns of *AtOPT8* expression in whole seedling (**h**) and hypocotyl (**i**) and in rosette leaf (**j**) and leaf petiole (**k**) of 25-day-old soil-grown plant. Note strong GUS staining only in primary veins of rosette leaves in (**h**). **l–o** Representative expression patterns of promoter–GUS fusions observed for *AtOPT1* (**l**), *AtOPT4* (**m, n**) and *AtOPT7* (**o**) in inflorescence stems. *Bars* = 2 mm (**a**, **f**, **h**, **j**, **k**, **l**, **m**), 150 μm (**b**, **c**, **d**, **e**, **g**, **i**), 300 μm (**n**, **o**)

AtOPT6 was strongly expressed in vascular tissues of sepals, petals, style, filaments, and ovary but no expression was detected in anthers and pollen grains (Fig.3i, j). The high expression of *AtOPT6* in unfertilized gynoecium was unique among the *AtOPT*s examined, especially expression in ovules (Fig. 3i). Following fertilization, a decrease in overall *AtOPT6* expression was observed in the gyneocium, although prominent expression in vascular tissues of the ovary and style was apparent (Fig 3j).

Similar to *AtOPT1* and *AtOPT3*, *AtOPT8* was strongly expressed in anthers and pollen grains starting at stage 12 of flower development (Fig. 3k). *AtOPT8* gave no detectable expression in ovules. However, strong *AtOPT8* expression was observed in ovules immediately following fertilization (Fig. 3l). *AtOPT8* gave no detectable expression in other floral organs, although weak GUS staining was observed in vascular tissues of sepals and filaments (data not shown).

In summary, notable differences in tissue-specific expression were observed for the *AtOPT*s in flowers before and after fertilization. *AtOPT1*, *AtOPT3* and *AtOPT8* were strongly expressed in anthers and pollen grains, whereas *AtOPT4*, *AtOPT6* and *AtOPT7* were not. In the unfertilized gynoecium, only *AtOPT6* was expressed in ovules and very strongly in vascular tissues. Only *AtOPT1* was expressed along the septum of ovary,

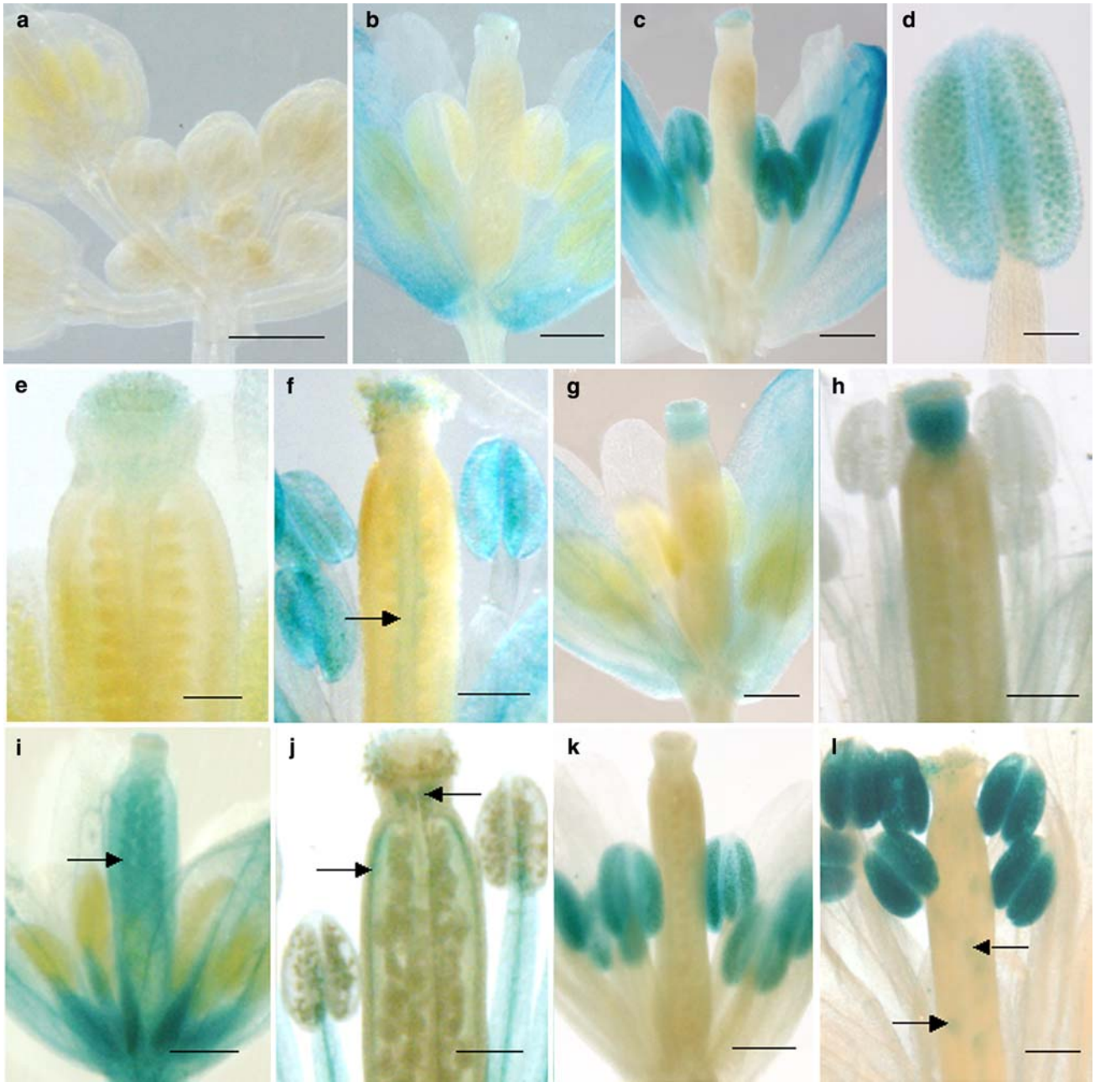

Fig. 3 a–l Histochemical localization of *AtOPT* promoter–GUS expression during *Arabidopsis* flower development and fertilization. **a–e** Representative expression patterns of *AtOPT1* promoter–GUS expression in immature flowers and floral buds (**a**) and in unfertilized flowers at stage 12 of floral development (**b–e**). At stage 12, *AtOPT1* was expressed in pollen grains (**c, d**) but not in ovules (**e**). **f** *AtOPT1* expression in fertilized flower at stage 15. *Arrow* indicates GUS staining along the septum of the ovary. **g-l** Representative expression patterns of promoter–GUS fusions observed for *AtOPT4* (**g, h**), *AtOPT6* (**i**, **j**) and *AtOPT8* (**k**, **l**) in unfertilized (**g**, **i**, **k**) and fertilized (**h**, **j**, **l**) flowers. *Arrows* in (**i**) and (**j**) indicate *AtOPT6* expression in ovules and vascular tissue of ovary, respectively. *Arrows* in (**l**) indicate *AtOPT8* expression in ovules immediately following fertilization. Unfertilized flowers were at stage 12 and fertilized flowers were at stages 14–15. The stages of flower development were as described by Smyth et al. (1990). *Bars* = 300 μm (**a**, **f**, **h**, **j**, **l**), 150 μm (**b**, **c**, **g**, **i**, **k**), 100 μm (**d**, **e**)

while only *AtOPT8* was expressed in fertilized ovules immediately following anthesis.

AtOPT1 is expressed in pollen tubes

The expression of *AtOPT1* along the septum of the ovary may be due to its expression in the maternal transmitting tract tissue or in the growing pollen tubes. To address this issue, stained pistils expressing the *AtOPT1* promoter–GUS fusion were cleared with lactophenol and examined in more detail (Fig. 4a–c). *AtOPT1*–GUS staining in the stigma and style appeared to be consistent with pollen tube expression as indicated by GUS-stained strands starting at stigmatic papillae

and through the central portion of the style (Fig. 4b). The *AtOPT1* expression pattern in the ovary was also consistent with pollen tube expression, being expressed in the central septum and in GUS-stained strands exiting the transmitting tract, which can be presumed to be pollen tubes penetrating ovules (Fig. 4c). Reciprocal crosses between Col-O and plants expressing an *AtOPT1* promoter–GUS fusion were performed to confirm that *AtOPT1* was indeed expressed in pollen tubes. When *AtOPT1* promoter–GUS plants were used as pollen donor, GUS staining was observed in the stigma and in the central septum of the ovary (Fig. 4d). When Col-O plants were used as pollen donor, no staining was observed in the stigma and the central septum (Fig. 4e). Control crosses, where *AtOPT1* promoter–GUS plants were manually self-fertilized, showed the expected GUS staining in stigma and central septum (Fig. 4f). Therefore, *AtOPT1* was expressed in pollen tubes and not in transmitting tract tissues during pollination.

Expression of *Arabidopsis OPT*s during seed development

The developing seed is a major sink tissue that requires nutrients from other parts of the plant. *AtOPT* expression patterns were observed during various stages of seed development, namely, following anthesis until seed desiccation, to determine if peptide transport through AtOPTs plays a role in seed development. Only *AtOPT8* was expressed in fertilized ovules immediately following anthesis and, therefore, *AtOPT8* expression was examined in more detail. *AtOPT8*–GUS staining was seen in cleared siliques and excised seeds (Fig. 5a–e). *AtOPT8* was expressed in endosperm and integument tissues of seeds at the preglobular (Fig. 5a, b) and globular stages (Fig. 5c) of seed development. By early heart stage, no *AtOPT8* expression in the endosperm and integument was observed, but rather *AtOPT8* expression was only observed in the funiculus (Fig. 5d). No *AtOPT8* expression was observed in embryos in all stages of seed development examined. Under similar staining conditions as those used for *AtOPT8*, no detectable seed expression was observed for *AtOPT7* during early embryogenesis (Fig. 5f–h). No detectable expression was also observed for *AtOPT1*, *AtOPT4* and *AtOPT6* during early embryogenesis (data not shown). However, like *AtOPT8*, these *AtOPT*s were strongly expressed in the vascular tissues of funiculi starting at around the onset of heart stage of embryogenesis, as shown in Fig. 5i for *AtOPT7*. All the *AtOPT*s were also expressed in the vascular tissues of siliques, with stronger expression observed starting at the heart stage until the curled cotyledon stage of embryogenesis, as shown in Fig. 5e

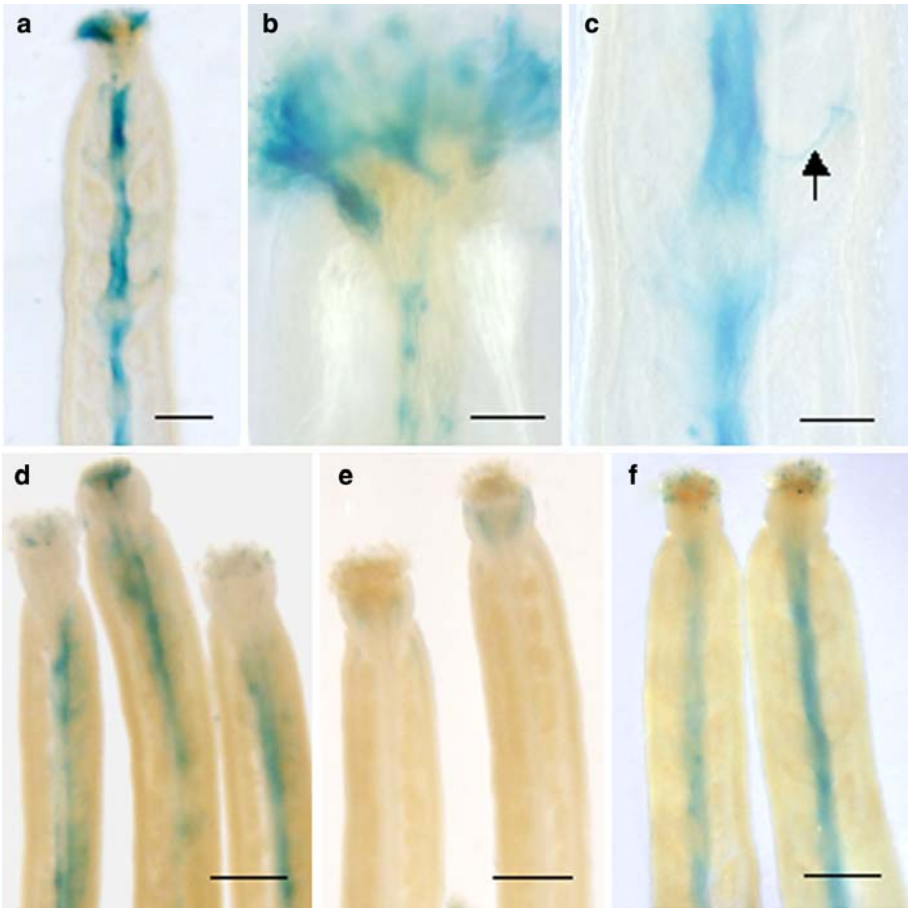

Fig. 4 **a–c** Expression of *AtOPT1* promoter-GUS in pollen tubes as indicated by GUS staining patterns in cleared silique (**a**), stigma and style (**b**) and ovary (**c**). *Arrow* in (**c**) indicates stained pollen tube exiting the transmitting tract tissue. **d** GUS-stained Col-O siliques fertilized with pollen grains from plants expressing *AtOPT1* promoter–GUS. **e** GUS-stained siliques from plants expressing *AtOPT1* promoter–GUS fertilized with Col-O pollen grains. **f** GUS-stained siliques from manually selfed plants expressing *OPT1* promoter-GUS. *Scale bars* = 500 μm (**a**, **d–f**), 60 μm (**b**, **c**)

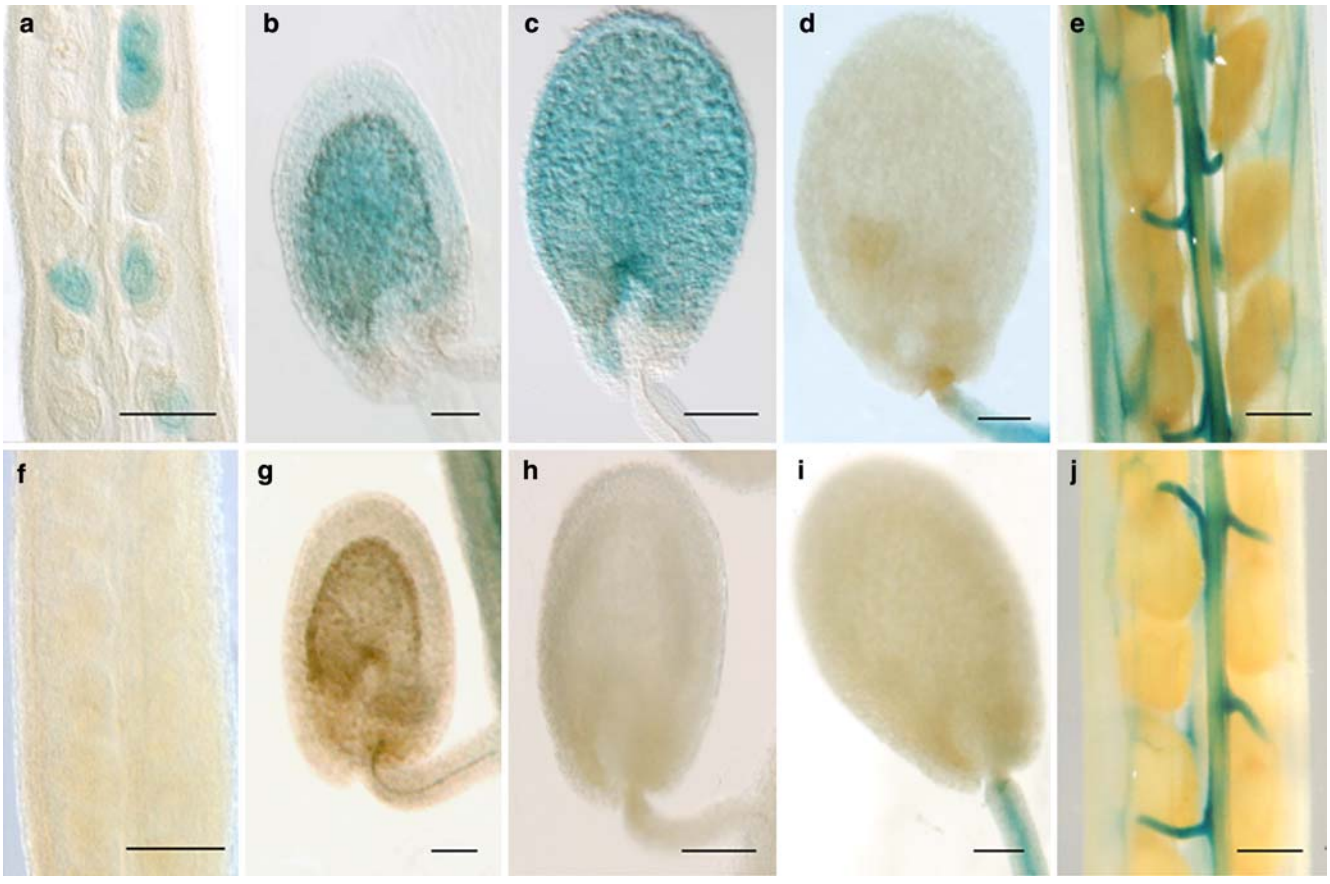

Fig. 5 a–j Histochemical localization of *AtOPT8* and *AtOPT7* promoter–GUS expression during seed development. Whole siliques or excised seeds were stained for GUS and subsequently cleared to visualize *AtOPT8* (**a–e**) or *AtOPT7* (**f–j**) expression at various stages of seed development. GUS-staining patterns were observed in siliques immediately following anthesis (**a**, **f**) and in seeds at the preglobular (**b**), globular (**c**, **g**, **h**), heart (**d**, **i**), and torpedo (**e**, **j**) stages of seed development. *Scale bars* = 300 μm (**a**, **e**, **f**, **g**), 60 μm (**b–d**, **g–i**)

and j for *AtOPT8* and *AtOPT7*, respectively. Lastly, as we previously reported, *AtOPT3* was expressed in developing seeds and embryos (Stacey et al. 2002b). Further examination of developing seeds showed that increased *AtOPT3* expression in vascular tissues of funiculi and siliques also occurred around the onset of the heart stage of embryogenesis (data not shown).

Corrected annotation for the *AtOPT3* genomic sequence

We recently described an embryo lethal phenotype for *opt3* plants (Stacey et. al. 2002b). AtOPT3 showed the highest sequence similarity among the nine AtOPTs to BjGT1, a recently characterized glutathione (GSH) transporter in *Brassica juncea* (Bogs et al. 2003). Bogs et al. (2003) also suggested that the predicted protein derived from the annotated *AtOPT3* gene (At4g16370) displays an erroneous C-terminal sequence, and proposed a sequencing error in the At4g16370 genome sequence. This conclusion was supported by comparison of BjGT1 and AtOPT3 with a full-length Col-O cDNA sequence (AY054590) derived from the *AtOPT3* gene (Bogs et al. 2003). As a first step for further studies on possible functions of AtOPT3, we re-isolated an *AtOPT3* cDNA from Col-O. Our cDNA sequence was identical to that of the AY054790 nucleotide sequence except for a single nucleotide difference at position 133 (A in AtOPT3 and G in AY054790), translating into an amino acid difference at position 45 (T in AtOPT3 and A in AY054790). However, our cDNA sequence corresponded to the nucleotide and predicted amino acid sequence to the annotated At4g16370 at this position and to the amino acid sequence of BjGT1 (Bogs et al. 2003). Our cDNA sequence also supported the previous suggestion of a sequencing error at the 3′ end of At4g16370. An additional G in At4g16370 at position 12378 causes a frameshift in the annotated amino acid sequence and a premature stop codon 93 base pairs upstream of the actual stop codon.

Previous data showed that *S. cerevisiae* expressing AtOPT3 was unable to mediate the uptake of glutathione ([H^3]-GSH) or of several peptides that were obtained with the truncated Ler *AtOPT3* cDNA based on the annotated At4g16370 sequence (Koh et al. 2002). Therefore, we re-evaluated the glutathione (GSH) and peptide transport capacities of AtOPT3 by expressing the full-length Col-O cDNA in *Xenopus laevis* oocytes or

S. cerevisiae. As control, we also used ScOPT1p (Hauser et al. 2000), previously shown to be a GSH transporter in *S. cerevisiae* (Bourbouloux et al. 2000). Oocytes expressing ScOPT1 exhibited GSH-evoked inward current when substrate was added in the bath solution at pH 5.0. However, oocytes expressing *AtOPT3* did not show measurable changes in the presence of GSH in the bath solution (data not shown). Likewise, no evidence for GSH or peptide (YGGFL, KLGL and KLLLG) uptake was found in yeast cells expressing AtOPT3 (data not shown). It is possible that AtOPT3 functions in peptide efflux rather than uptake. However, transporters in this class are generally reversible. The direction of substrate movement would then depend on the electrochemical gradient, which in uptake experiments favors substrate influx. Additional experiments are required to establish true substrates and the transport mechanism of AtOPT3.

Expression of *AtOPT3* is induced by limiting iron

Gene expression of other OPT family members, for example, the maize YS1 and the *Arabidopsis* YS-like proteins (YSLs), was shown to be regulated by iron availability in the growth medium (Curie et al. 2001; DiDonato et al. 2004). Therefore, the expression of *AtOPT*-GUS fusions was also examined in seedlings grown under iron-sufficient and iron-deficient conditions. As shown in Fig. 6a, *AtOPT3* expression was induced two- to fourfold under iron-deficient conditions. *AtOPT3* expression was increased in rosette leaves and root tissues under limiting iron (Fig. 6b). Increased *AtOPT3* expression in leaves was observed mostly in younger leaves (sixth to tenth leaf) and was not limited to vascular tissues. In roots, increased *AtOPT3* expression under iron-deficient conditions was observed predominantly in vascular tissues. No detectable *AtOPT3* expression was observed in root hairs and root tips under both iron-sufficient or iron-deficient conditions (Fig. 6b). Histochemical staining showed no effect on the expression of *AtOPT1*, *4*, *6*, *7*, and *8* under limiting-iron availability (data not shown) and this was confirmed by GUS assays as shown in Fig. 6a.

Examination of the abiotic stress microarray dataset from the AtGenExpress project (http://www.weigelworld.org/resources/microarray/AtGenExpress) showed no induction of *AtOPT* expression under the various stress conditions examined, except for a significant induction of *AtOPT2* under salt stress. We found no effect of salt stress (150 mM NaCl) on the expression *AtOPT*s, including *AtOPT2* data not shown. It is possible that the *AtOPT2* promoter fusion that we used lacked important regulatory regions responsive to salt stress.

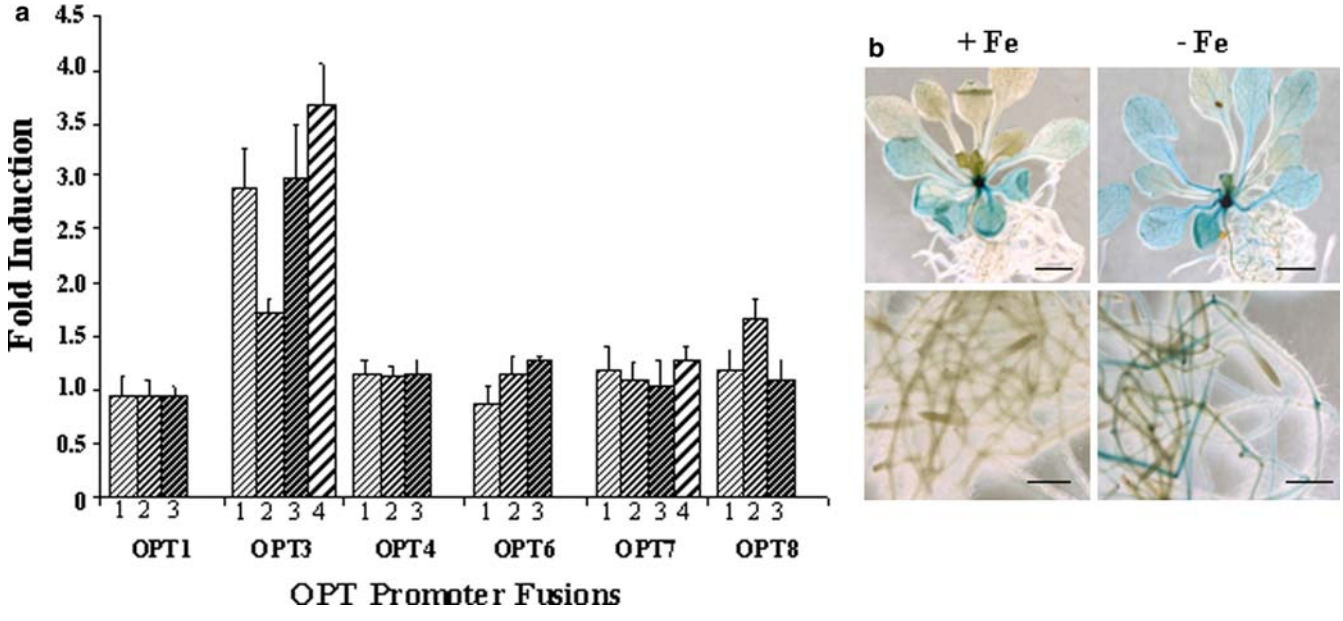

Fig. 6 **a** Quantitative GUS activity assays of transgenic *Arabidopsis* seedlings expressing various *AtOPT* promoter–GUS fusions using MUG as substrate. GUS activity in T2 whole seedlings was measured after growing plants for 5 days in iron-sufficient (50 μM Fe(III)–EDTA) or iron-deficient medium. For each *AtOPT* promoter–GUS fusion, three or four independent lines were assayed (indicated by numbers on *x*-axis). Values represent average relative ratio of GUS activity in iron-deficient plants to the GUS activity of iron-sufficient plants (fold induction). For each transgenic line, GUS activity was measured in six to eight replicates performed over three trials. Standard error bars are shown. **b** GUS-stained plants expressing *AtOPT3* promoter-GUS grown for five days in iron-sufficient (+FE) or iron-deficient (−Fe) medium. *Scale bar* = 2 mm (**b**, *upper panels*), 0.5 mm (**b**, *lower panels*)

Discussion

AtOPTs may function in nitrogen mobilization during seed germination and senescence

One proposed function for peptide transport in plants is the mobilization of peptides derived from proteolyzed

storage proteins during germination (Higgins and Payne 1981, 1982; reviewed by Waterworth et al. 2001). For example, physiological and biochemical analyses of storage protein degradation in germinating cereal seeds showed that storage proteins in the endosperm are hydrolyzed to form a reservoir of small peptides (two to six residues) and amino acids, which are subsequently transported across the scutellum to the growing embryo (Higgins and Payne 1981). To examine if the AtOPTs are involved in nitrogen mobilization during germination and subsequent early seedling growth, the expression of *AtOPT*s was examined to see if it coincided temporally and spatially with storage protein degradation during these early stages of plant growth. Germination is regarded to be complete when the radicle breaks through the seed coat after which the onset of seedling growth begins (Bewley 1997). Proteolysis of storage proteins prior to radicle emergence was reported in barley (Higgins and Payne 1981), vetch, garden beans and rape seeds (Schlereth et al. 2001; Tiedemann et al., 2000; reviewed by Müntz et al. 2001). Consistent with this timing, *AtOPT1*, *AtOPT3*, *AtOPT6* and *AtOPT7* showed detectable GUS staining in embryonic cotyledons prior to radicle emergence. Likewise, the prominent expression of AtOPTs in prevascular tissues of germinating seeds, and subsequently in vascular tissues of postgerminative seedlings, is of significance with regard to their potential role in nitrogen mobilization during the germination process. In germinating vetch and garden bean seeds, the prevascular strands are among the first tissues where protein mobilization is initiated during germination (Schlereth et al. 2001; Tiedemann et al. 2000). Local proteolysis is initiated in these tissues presumably to support growth and differentiation prior to the formation of functional vascular bundles (Schlereth et al. 2001; Tiedemann et al. 2000). Bulk storage protein degradation in the cotyledons, where most stored nutrients are located, occurs during the postgerminative period (Müntz et al. 2001).) Thus, in germinating *Arabidopsis* seeds, most of the AtOPTs are likely involved in the transport of peptides derived from storage protein degradation in cells undergoing growth and differentiation and, subsequently, in the mobilization of peptides from the cotyledons to the embryonic axis during postgerminative growth. It is curious, however, that no detectable *AtOPT* expression was seen in root tissues, which also have protein reserves that are mobilized during germination (Tiedemann et al. 2000).

Senescence in plants is characterized by an ordered degradation of cell constituents and functions mainly for mobilization and recycling of nutrients (reviewed by Hörtensteiner and Feller 2002; Buchanan-Wollaston et al. 2003). Significant levels of peptides were reported in phloem and xylem exudates and peptide transport was suggested to be important for bulk movement and redistribution of organic nitrogen during senescence (Higgins and Payne 1980; Higgins and Payne 1982). Ribulose-1,5-bisphosphate carboxylase (Rubisco) accounts for at least 50% of total soluble protein in a fully expanded leaf and was suggested to be an important nitrogen storage component that could be rapidly mobilized during leaf senescence (Mae et al. 1983). *Arabidopsis* leaves have a very short lifetime. Senescence starts as soon as full expansion is reached, as indicated by diminished levels of Rubisco large subunit as soon as leaf expansion is complete (Stessmann et al. 2002; Buchanan-Wollaston et al. 2003). The fact that none of the *AtOPT*s were expressed in the root epidermis or root hairs, argues against a role for these transporters in peptide acquisition from the growth medium. Rather, their expression patterns support the idea that AtOPTs mediate the long-distance mobilization of endogenous peptides, possibly derived from proteolysis in senescing older leaves. This hypothesis is consistent with the prominent expression of *AtOPT*s in the vascular tissue of source tissues, especially in cotyledons and in older leaves.

In addition to the AtOPTs, peptide transporters belonging to the PTR family were also proposed to be important for the mobilization of nitrogen during germination and in the long-distance transport of peptides to growing tissues. These include the barley HvPTR1 (West et al. 1998; Waterworth et al. 2000), the Faba bean VfPTR1 and VfPTR2 (Miranda et al. 2003) and the *Arabidopsis* AtPTR1 (Dietrich et al. 2004).

Differential expression of *AtOPT*s during flower and seed development indicates distinct roles for specific *AtOPT*s

Unlike vegetative growth where only minor differences in tissue-specific expression among the various *AtOPT*s were observed, major differences in *AtOPT* expression were observed during flower and seed development (Table 1). *AtOPT1*, *3* and *8* were strongly expressed in anthers and pollen grains, whereas *AtOPT4*,*6* and *7* were not. *AtOPT1*, *3* and *8* were expressed late in pollen development, starting at stage 12 of anther development right before anther dehiscence and the opening of floral buds (Smyth et al. 1990; Sanders et al. 1999). These data appear to rule out an important role for these AtOPTs in the early events of anther development. Rather, the timing of *AtOPT* expression suggests a role in anther dehiscence and/or during pollination. AtOPT1 and AtOPT6 are the only peptide transporters shown so far to be expressed in pollen tubes and ovules, respectively. AtOPT1 is likely involved in the transport of peptides from the surrounding maternal transmitting tract tissues for use in nourishing pollen tube growth. Likewise, AtOPT6 may also have a nutritional function during ovule development.

Transgenic plants expressing promoter-GUS fusions to *AtOPT2*, *AtOPT5* and *AtOPT9* showed little to no expression (data not shown) and hence, detailed expression analyses for these genes were not pursued. Massively parallel signature sequencing (MPSS) data

Table 1 Tissue-specific expression of *AtOPT* promoter–GUS fusions during flower and seed development

Gene	Unfertilized flowers (stage 12)			Siliques/seeds
	Petal/sepal	Gynoecium	Antheridium	
AtOPT1	Vascular	Style Stigma	**Pollen grains** Filament	**Pollen tubes** Vascular
AtOPT3	Vascular	Style Filament	**Pollen grains** **Anther**	Vascular **Endosperm** **Integuments** **Embryo**
AtOPT4	Vascular Stigma	Style	Filament	Vascular
AtOPT6	Vascular	**Carpel** **Ovules** Stigma	Filament Style	Vascular
AtOPT7	Vascular	Style Stigma	Filament	Vascular
AtOPT8	Vascular (weak)	None	**Pollen grains** **Anther** Filament	Vascular **Endosperm** **Integuments**

Summary of *AtOPT* promoter-GUS expression patterns from data shown in Figs. 3, 4, 5. Expression of AtOPT3 was based on previously published data (Stacey et al. 2005b). Vascular denotes predominant, but not exclusive, expression in vascular tissues. *AtOPT* expression in style and filament was restricted to vascular tissues. *AtOPT* expression in endosperm and integuments was

also indicated very low expression levels for *AtOPT2* and *AtOPT9*. However, examination of the developmental microarray dataset (Schmid et al. 2005) showed high expression of *AtOPT2* and *AtOPT9* in young flowers (at stages 9 and 10 of flower development), as well as in stamen and pollen grains. *AtOPT5* also showed significant expression in floral organs of fertilized and unfertilized flowers. Unlike *AtOPT2* and *AtOPT9*, however, *AtOPT5* showed a significant expression in developing seeds, as well as in vegetative tissues of seedlings and adult plants. The specificity of *AtOPT2* and *AtOPT9* expression in flowers could indicate an important function in flower development. Although, in general, our GUS expression data fit well with the AtGenExpress data (Schmid et al. 2005), the use of the promoter–GUS fusions allowed a more detailed examination, involving a wider variety of conditions and developmental stages, than practical with DNA microarray technology.

Immediately following fertilization up to the globular stage, *AtOPT8* is expressed in integument and endosperm tissues of developing seeds. At around the start of heart stage, *AtOPT8* expression was observed only in seed funiculi. A similar expression pattern was observed for *AtOPT3* in developing seeds, except that, unlike *AtOPT8*, it was expressed in developing embryos (Stacey et al. 2002b). This pattern of expression indicates that AtOPT3 and AtOPT8 are likely involved in the apoplastic transport of peptides between integument cells and the symplastically isolated endosperm during early embryogenesis. At the heart stage of embryogenesis, the start of endosperm cellularization, rapid embryo growth and deposition of storage proteins in embryonic cotyledons create a high demand for nitrogen assimilates. Moreover, at the heart stage of embryo development, a specialized maternal tissue, the chalazal proliferative tissue, is formed in close proximity to the vascular tissue of the funiculus and was proposed to facilitate solute transfer across the plasmalemma between the maternal plant and the apoplastic endosperm (Olsen 2004; Brown et al. 1999). The timing of *AtOPT3* and *AtOPT8* expression, as well as that of other *AtOPT*s, in the vascular tissues of funiculi starting at around the onset of the heart stage of embryogenesis is highly suggestive of their functional role in the vascular transport of nitrogen assimilates from the maternal plant to the developing seeds. Vascular transport of peptides and other assimilates during later stages of embryogenesis could be a more efficient way to meet higher nitrogen demand during these stages. Consistent with this notion, the *Arabidopsis* amino acid (AAP2) transporter was also strongly induced at the heart stage of embryo development in the vascular strands of siliques and funiculi (Hirner et al. 1998). It is very likely that AtOPT5 is also involved in nitrogen mobilization during seed development, as indicated by its relatively high expression in young siliques and developing seeds (AtGenExpress developmental microarray data; Schmid et al. 2005).

Non-protein-derived peptides as substrates of AtOPTs

The OPT family includes the maize Yellow-Stripe1 (YS1) protein involved in the uptake of Fe^{3+} when complexed with mugeneic acids (MAs) (Yen et al. 2001; Curie et al. 2001), peptide-like compounds synthesized from three molecules of *S*-adenosyl methionine. *Arabidopsis* and other dicots do not produce MAs but

synthesize a structurally similar compound, nicotianamine (NA), the biosynthetic precursor of MAs. YS1-like proteins (YSLs) have been characterized in *Arabidopsis* (AtYSL2; DiDonato et al. 2004) and rice (OsYSL2; Koike et al. 2004). AtYSL2 and OsYSL2 were shown to transport metal–NA complexes and their expression was influenced by the availability of iron in the growth medium. On the basis of their *in planta* expression patterns, both proteins were proposed to transport internal metal–NA complexes, rather than metal acquisition from the soil, as is the case with ZmYS1. The YSL subfamily of OPT transporters is phylogenetically distinct from the AtOPT transporters (Yen et al. 2001). However, Wintz et al. (2003) showed that *AtOPT3* expression was also influenced by the availability of iron in the growth medium and that AtOPT3 mediated the transport of copper, manganese and iron when expressed in yeast. Consistent with these earlier findings, we found that *AtOPT3* promoter–GUS expression was inducible by iron limitation. Limiting iron conditions had no effect on the expression of *AtOPT1*, *4*, *6*, *7* and *8*. The preferential expression of *AtOPT3* in vascular tissues of major plant organs and its lack of expression in root hairs suggest that AtOPT3, like the *Arabidopsis* and rice YSLs, is likely involved in internal metal–chelate transport rather than in metal acquisition from the external environment. If this hypothesis is correct, then the expression of *AtOPT3* in the embryo and the embryo-lethal phenotype of an *opt3* mutation argue for a major role for AtOPT3-mediated metal transport or efflux of metal chelators during embryo development. Other members of the OPT family also mediate the uptake of non-protein-derived peptides such as glutathione (GSH and GSSG) and glutathione derivatives (Bourbouloux et al. 2000; Bogs et al. 2003; Zhang et al. 2004; Cagnac et al. 2004). Expression of AtOPT6, but not AtOPT7, has recently been shown to be inducible by ABA and the sulfonylurea herbicide primisulfuron (Cagnac et al. 2004). It has been suggested that this transporter may be involved in detoxification and/or stress resistance, including metal stress. It remains to be tested if the expression of other AtOPTs is also affected by primisulfuron.

In summary, careful analysis of the expression patterns of *AtOPT* promoter–GUS fusions during various stages of *Arabidopsis* growth and development has led to testable hypotheses regarding transporter function. In general, *AtOPT* expression in vascular tissues, especially in source tissues such as cotyledons and older leaves, suggests a role in long distance movement of peptides resulting from proteolytic breakdown of storage protein. This role is also reflected in the germinating seed where AtOPTs likely mobilize protein reserves for new growth. The lack of AtOPT expression in the root epidermis and root hairs argues against a role in mediating peptide uptake from the medium. *AtOPT1* expression in pollen tubes suggests a role in transport of peptides from the maternal tissue during fertilization. *AtOPT3* and *AtOPT8* showed a developmentally regulated pattern of expression in developing seeds. The data suggest a role for these transporters in aiding embryo nutrition from endosperm reserves until the heart stage. Subsequently, AtOPT expression switches to the vascular tissue of the funiculus, concomitant with the development of the funiculus-mediated pathway for maternal nutrition of the developing seed. Finally, although AtOPT3 is divergent in sequence from members of the YSL subfamily of OPT transporters, its expression is enhanced under limiting iron and studies in yeast suggest a role in metal uptake. Therefore, this transporter is likely involved in long-distance movement of metal–chelate complexes in the plant. This role appears especially critical, given the embryo-lethal phenotype of an *opt3* mutant.

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