AtOPT6 Transports Glutathione Derivatives and Is Induced by Primisulfuron[1]

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The oligopeptide transporter (OPT) family contains nine members in Arabidopsis. While there is some evidence that AtOPTs mediate the uptake of tetra- and pentapeptides, OPT homologs in rice (*Oryza sativa*; OsGT1) and Indian mustard (*Brassica juncea*; BjGT1) have been described as transporters of glutathione derivatives. This study investigates the possibility that two members of the AtOPT family, AtOPT6 and AtOPT7, may also transport glutathione and its conjugates. Complementation of the *hgt1met1* yeast double mutant by plant homologs of the yeast glutathione transporter HGT1 (AtOPT6, AtOPT7, OsGT1, BjGT1) did not restore the growth phenotype, unlike complementation by HGT1. By contrast, complementation by *AtOPT6* restored growth of the *hgt1* yeast mutant on a medium containing reduced (GSH) or oxidized glutathione as the sole sulfur source and induced uptake of [^{3}H]GSH, whereas complementation by *AtOPT7* did not. In these conditions, AtOPT6-dependent GSH uptake in yeast was mediated by a high affinity (K_m = 400 μM) and a low affinity (K_m = 5 mM) phase. It was strongly competed for by an excess oxidized glutathione and glutathione-*N*-ethylmaleimide conjugate. Growth assays of yeasts in the presence of cadmium (Cd) suggested that AtOPT6 may transport Cd and Cd/GSH conjugate. Reporter gene experiments showed that *AtOPT6* is mainly expressed in dividing areas of the plant (cambium, areas of lateral root initiation). RNA blots on cell suspensions and real-time reverse transcription-PCR on Arabidopsis plants indicated that *AtOPT6* expression is strongly induced by primisulfuron and, to a lesser extent, by abscisic acid but not by Cd. Altogether, the data show that the substrate specificity and the physiological functions of AtOPT members may be diverse. In addition to peptide transport, AtOPT6 is able to transport glutathione derivatives and metal complexes, and may be involved in stress resistance.

As other eucaryotic cells and procaryotes, plant cells have the ability to transport peptides across membranes. Peptide transport is important for storage and mobilization of reduced nitrogen (Higgins and Payne, 1977), and it is also involved in a wide range of cellular processes, including quorum sensing in bacteria, yeast mating, and the immune response in mammals (Stacey et al., 2002b). Furthermore, peptide-derived antibiotics (Dantzig et al., 1992) and anticancer agents (Hori et al., 1993) are also transported by peptide transporters.

The presence of multiple peptide transporters in the Arabidopsis genome and the known functions of peptide transport in bacteria, fungi, and animals suggest that peptide transporters may also play a key role in plant growth and development (Stacey et al., 2002a). Three gene families have been shown to transport various peptides in Arabidopsis, i.e. the peptide transporter (PTR) family, the oligopeptide transporter (OPT) family, and the multidrug resistance-associated protein (MRP) family.
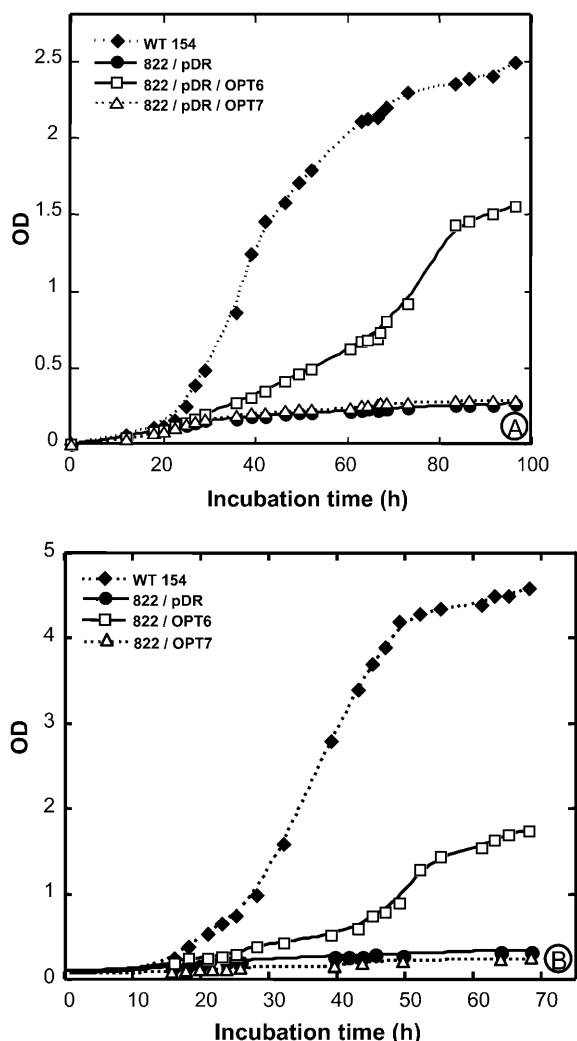
The Arabidopsis genome contains 51 PTR family members (Stacey et al., 2002a). AtPTR2, the best known member of the family, mediates a cotransport of protons with dipeptides and tripeptides but not with larger peptides (Steiner et al., 1994, 1995; Song et al., 1996). AtPTR2 antisense plants exhibit delayed flowering and are arrested in seed maturation (Song et al., 1997). The Arabidopsis genome also contains nine members of the OPT family that are genetically and physiologically distinct from the PTR proteins (Koh et al., 2002). The AtOPT members encode proteins predicted to have 12 to 14 transmembrane domains and exhibit two conserved motifs (NPG and KIPPR) in protein regions predicted to be hydrophilic (Koh et al., 2002). Growth studies with a yeast Leu auxotroph mutant complemented by the AtOPT members indicated that seven members of the family (all but AtOPT2 and 3) transport tetra- and pentapeptides, presumably with proton cotransport (Koh et al., 2002). OPT homologs have also been characterized in other plants and in yeast. The *Saccharomyces cerevisiae* OPT1 protein was reported to transport the pentapeptides YGGFL and YGGFM (Leu- and Met-enkephalin; Hauser et al., 2000), reduced glutathione (GSH), oxidized glutathione

Figure 1. Complementation of the ABC 822 (*hgt1Δ*) strain by *AtOPT6* and *AtOPT7*. A, Growth assay in the presence of 500 μM GSH. The ABC 822 strain transformed with either the empty vector pDR or the pDR/*AtOPT6* and *AtOPT7* construct were grown in SD medium to OD_{600} = 0.6, washed three times in cold sterile water, and diluted to OD_{600} = 0.001 in SD-S medium containing 500 μM GSH. Their growth was compared with that of the wild-type strain ABC 154 under the same conditions. B, Growth assay in the presence of 500 μM GSSG.

(GSSG), and glutathione conjugates (Bourbouloux et al., 2000). Likewise, *BjGT1*, an OPT member from Indian mustard (*Brassica juncea*), and OsGT1, an OPT homolog from rice (*Oryza sativa*), were shown to transport GSSG and GS conjugates (Bogs et al., 2003; Zhang et al., 2004). In this respect, the OPT family resembles the last family of Arabidopsis peptide transporters, AtMRP1-4, which mediates the transport of GS conjugates (Lu et al., 1997, 1998; Sanchez-Fernandez et al., 1998). In terms of substrate specificity, the OPT family thus seems to combine properties of the PTR family, which is specific for di- and tripeptides, and of the MRP family, which is specific for GS derivatives.

However, although the ability of Arabidopsis OPT members to transport tetra- and pentapeptides has

been well established, the possibility that they may also transport glutathione and its derivatives has not yet been investigated in detail. This study demonstrates that AtOPT6 is able to transport glutathione in strains grown under sulfur-deprived conditions, whereas AtOPT7 is not. Furthermore, it is shown that expression of *AtOPT6*, which was characterized by β-glucuronidase (GUS) reporter gene experiments, RNA blots, and real-time reverse transcription (RT)-PCR, is sensitive to the herbicide primisulfuron and to abscisic acid (ABA). These data shed new light on the possible physiological roles of the AtOPT family and suggest that AtOPT6 may be involved in stress resistance.

RESULTS

AtOPT6 and *AtOPT7* full-length cDNAs were amplified from whole Arabidopsis seedlings by RT-PCR with specific primers and transferred to the yeast shuttle vector pDR 195 or pDR 196, respectively. The pDR/*AtOPT6* and *AtOPT7* constructs were used to transform the ABC 822 (*MATa ura3-52 leu2-Δ1 lys2-801 his3- Δ200 trp1- Δ63 ade2-101 hgt1Δ::LEU2*) and the ABC 817 yeast (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hgt1Δ::LEU2*) yeast mutants deficient in glutathione transport. The requirement of the ABC 817 strain for a source of exogenous reduced sulfur is expected to be higher than that of ABC 822 due to disruption of a gene essential for Met synthesis.

We first assessed the effects of complementation of ABC 817 by either pDR/*AtOPT6*, pDR/*AtOPT7*, or TEF-*HGT1* by studying the growth phenotype on synthetic dextrose (SD) solid media containing different organic sulfur sources (Met, Cys, GSH) in the presence of sulfate in the medium. While the *hgt1met15*
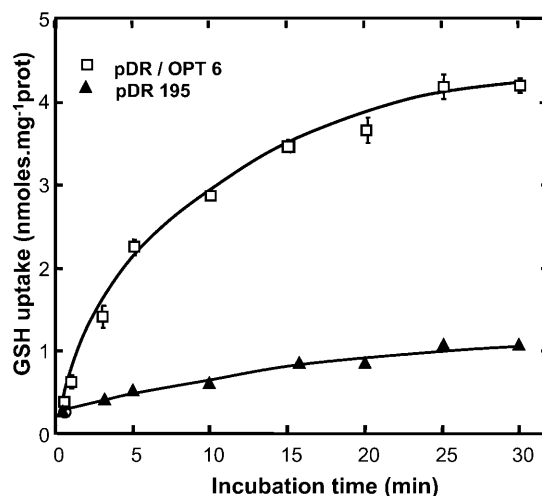
Figure 2. Time course of [³H]GSH uptake by the ABC 822 (*hgt1Δ*) strain transformed with either the empty pDR vector or the pDR/*AtOPT6* construct in a medium containing 100 μM [³H]GSH.
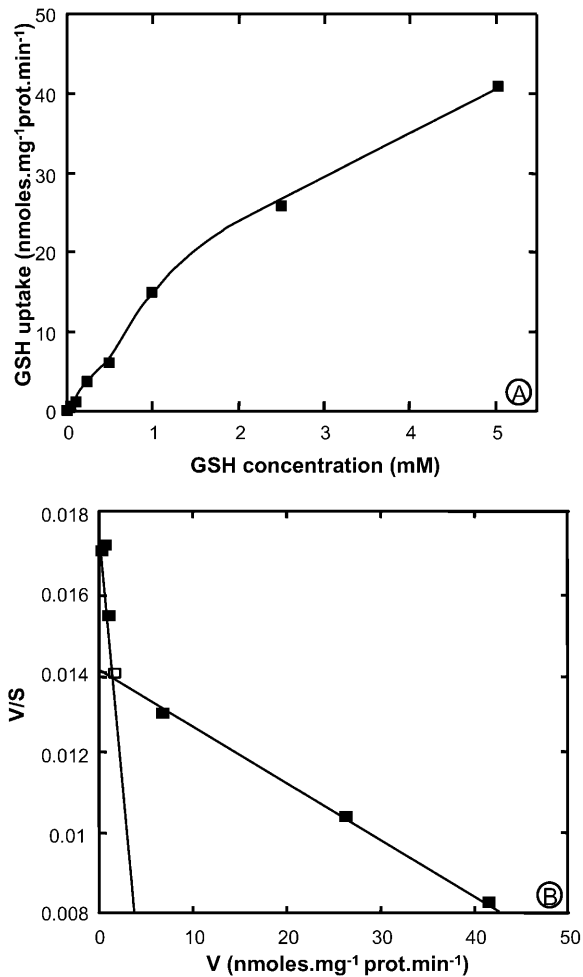
Figure 3. Kinetic analysis of [³H]GSH uptake by yeast strain ABC 822 (*hgt1*) expressing AtOPT6. Initial rates of uptake of GSH were measured with yeasts carrying either the pDR/*AtOPT6* construct or pDR alone. Background uptake measured with the strain carrying the empty plasmid was subtracted from uptake measured with the pDR/*AtOPT6* strain, and the data were plotted according to Michaelis-Menten (A) and Eadie-Hofstee (B). The latter representation yields straight lines of slope $-1/K_m$ and ordinal intercept V/K_m. V is given is nmol glutathione absorbed per min and per mg protein.

mutant complemented by the yeast glutathione transporter *HGT1* can grow in the presence of GSH, the transformants carrying either the *AtOPT6* or *AtOPT7* cDNA required the presence of either Met or Cys in the medium, but their growth was not restored by the presence of GSH alone. Similar results were obtained with the *hgt1met15* mutant complemented with *BjGT1* or *OsGT1* (data not shown). Furthermore, in the presence of Met, addition of high GSH concentrations (400 μM or 1 mM) did not affect the growth of the ABC 817 mutant complemented by the plant genes (compared to control without GSH), but it drastically inhibited the growth of the mutant carrying the TEF-*HGT1* construct (data not shown). These data indicate that in this genetic background and under those conditions, the ability of the plant genes to mediate

GSH transport in yeast was not sufficient, unlike that of the yeast transporter HGT1.

Further experiments were run with the ABC 822 strain, which, unlike the ABC 817 mutant, is affected in glutathione uptake, but not on Met synthesis. The ability of AtOPT6 to mediate uptake of glutathione was tested both by growth experiments and by direct measurements of glutathione uptake. The ABC 822 yeast strain disrupted on the *OPT1* (*HGT1*) gene did not grow on a sulfur-free minimal medium containing either GSH or GSSG as the sole sulfur source (Fig. 1). However, complementation of this strain by the pDR/*AtOPT6* construct allowed growth on the medium containing either GSH (Fig. 1A) or GSSG (Fig. 1B). Complementation of this strain by the empty vector or by the pDR/*AtOPT7* construct did not allow growth in the presence of GSH (Fig. 1A). Similar results were obtained for growth assays in solid media (data not shown).

After complementation by the pDR/*AtOPT6* construct, the ABC 822 strain was able to absorb labeled GSH, whereas it was not when transformed by the empty pDR vector. The uptake rate was constant during 5 min and thereafter decreased until 30 min (Fig. 2).

When the ABC 822 strain carrying the pDR/*AtOPT6* construct was grown in synthetic complete medium, which contains many sulfur compounds, uptake of [³H]GSH was very low (data not shown). Thus, the glutathione uptake capacity was observed only when the yeasts were grown in a sulfur-deprived medium (SD-S) where the only source of sulfur is glutathione, confirming previous results (Bogs et al., 2003; Zhang et al., 2004).
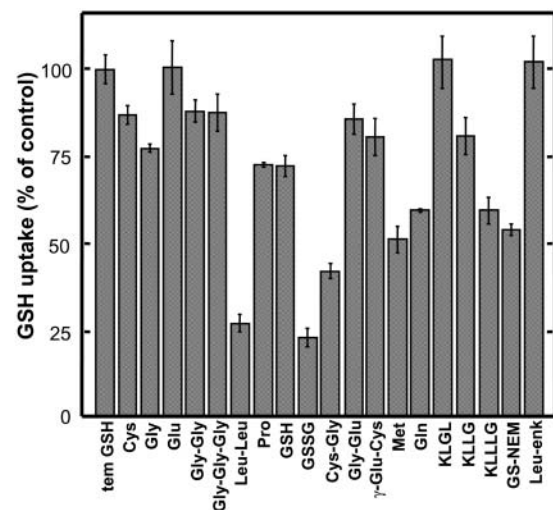
Figure 4. Effect of various compounds on GSH uptake in *hgt1*Δ/pDR/*AtOPT6* yeast. The initial rates of uptake from a 50 μM GSH solution were determined in the presence of various compounds (500 μM) as indicated. Data were plotted as % of control (net initial rate of GSH uptake). The control initial rate of GSH uptake was 561 pmol mg prot⁻¹ min⁻¹. Results are the mean ± SE of 8 to 12 samples from 2 or 3 independent experiments.

Concentration dependence studies showed that the initial rate of [³H]GSH uptake (measured within the first 3 min of incubation) was not fully saturable up to 5 mM (Fig. 3A). However, this rate decreased above 1 mM, and Eadie-Hostee plot analysis indicates that uptake kinetics may be interpreted as the superimposition of a low affinity (K_m = 5 mM) and a high affinity (K_m = 405 μM) component (Fig. 3B).

The substrate specificity of AtOPT6 was studied by competition experiments where the uptake of 50 μM [³H]GSH was challenged by a 10-fold excess of various potential competing substrates (Fig. 4). Various amino acids (Cys, Gly, Glu), dipeptides (Gly-Gly, Gly-Glu, γ-Glu-Cys), tripeptides (Gly-Gly-Gly), tetrapeptides (KLGL, KLLG), or the pentapeptide Leu-enkephalin (YGGFL) did not affect the uptake of [³H]GSH. By contrast, other amino acids (Pro, Met, Gln), dipeptides (Leu-Leu, Cys-Gly), and the pentapeptide KLLLG significantly decreased [³H]GSH uptake. Glutathione-N-ethylmaleimide conjugate and, even more, GSSG also decreased the uptake of labeled GSH. Addition of 500 μM unlabeled GSH significantly inhibited the uptake of 50 μM [³H]GSH, but the inhibition was not very strong, confirming that GSH did not completely saturate the transport system under these conditions. GSH uptake mediated by AtOPT6 in yeasts was strongly sensitive to protonophores and low temperature (data not shown).

At least five independent transgenic lines expressing either an *AtOPT6* promoter-GUS or *AtOPT7* promoter-GUS fusion were analyzed histochemically to study the pattern of AtOPT6 and AtOPT7 expression during development. In young embryos, a weak AtOPT6-GUS-driven expression transiently appeared in the procambium (Fig. 5A). At a later stage, GUS expression was apparent over the whole surface of the embryos (Fig. 5B). AtOPT6 was also strongly expressed in the inner tegument, in the area adjacent to the micropyle (Fig. 5C). In adult plants, AtOPT6 expression was detected in the vascular bundles of the leaves, petioles, and stems (Fig. 5D). In the petiole (Fig. 5G) and in the stem (Fig. 5H), GUS activity was the strongest in the cambial zone of the vascular bundles. In the roots, AtOPT6-driven GUS expression was noticed in the regions of lateral root initiation (Fig. 5, E and F). In the flowers, the expression was detected at a relatively low level in the vascular network of the petals; by contrast, the stamen filaments and the gynoecium were strongly stained (Fig. 5I).

AtOPT7-driven GUS expression was not detected at the embryo stage (Fig. 6, A and B). In adult plants, this expression was more important in the aerial parts than in the root system (Fig. 6C) and somewhat stronger than AtOPT6 expression, although this was not quantified. In the leaves, the expression was detected in the major and the first order veins (Fig. 6D) and in the hydathodes. In the roots, AtOPT7 was expressed in circular zones surrounding lateral root primordia (Fig. 6E) and in some parts of the root epidermis (Fig. 6F). Unlike AtOPT6, AtOPT7 was expressed in the cortical
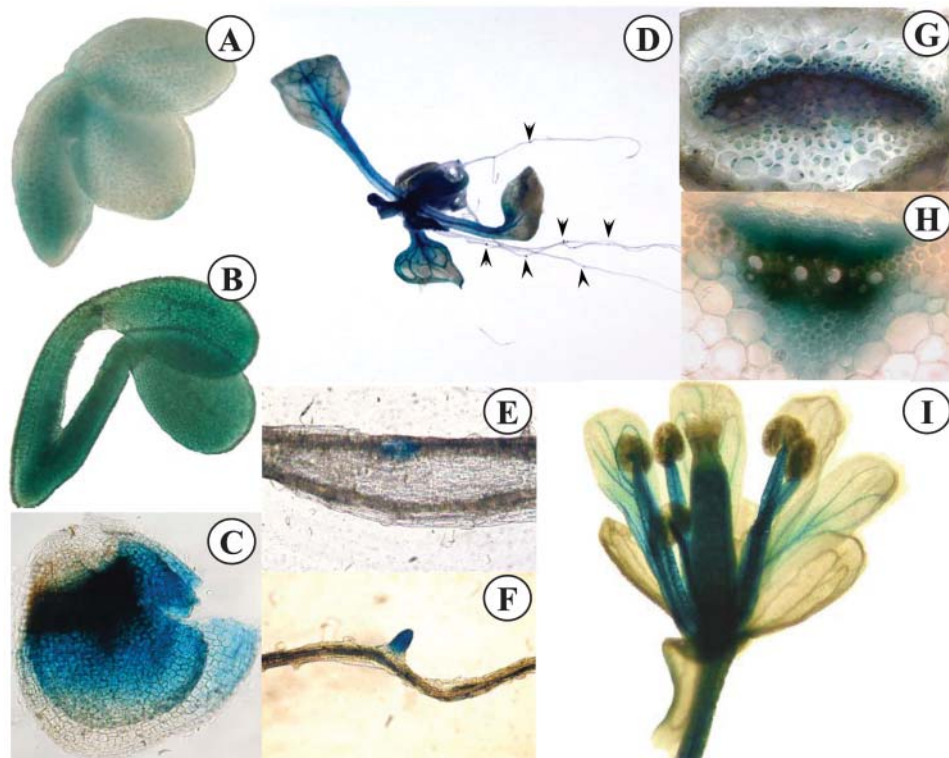
Figure 5. Histochemical localization of GUS activity in Arabidopsis transformed with the *AtOPT6* promoter region fused to the GUS reporter gene. A, Embryo; B, embryo; C, teguments left after embryo removal; D, whole plant; E and F, longitudinal views of roots; G, cross section of petiole; H, cross section of stem; I, flower.

Figure 6. Histochemical localization of GUS activity in Arabidopsis transformed with the *AtOPT7* promoter region fused to the GUS reporter gene. A, Embryo tegument; B, embryo; C, whole plant; D, leaf area; E and F, longitudinal views of roots; G, cross section of stem; H, flower.
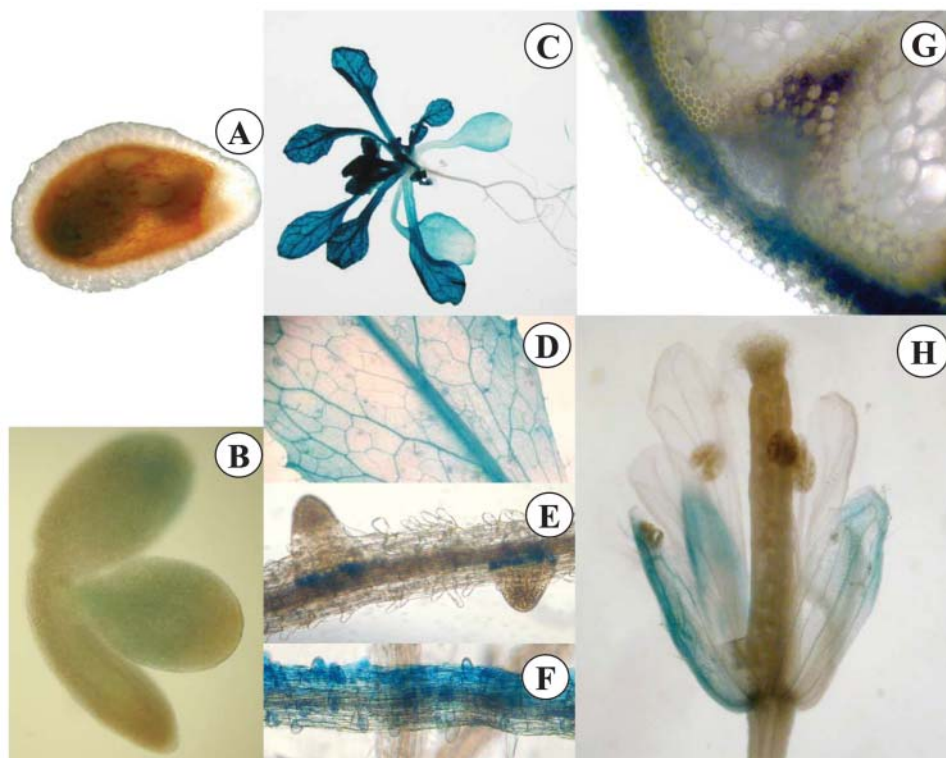

tissues of the stem but not in the conducting bundles (Fig. 6G). In the flowers, AtOPT7 was expressed at a low level in the sepals, but neither in the petals nor in the reproductive tissues (Fig. 6H).

Attempts to detect AtOPT6 expression by northern blot in Arabidopsis plants grown in normal conditions were unsuccessful (data not shown). The inductibility of AtOPT6 expression by various compounds was tested in Arabidopsis suspension cells. Because glutathione has been reported to be involved in resistance against biotic and abiotic stress, and more particularly against oxidative stress (Noctor and Foyer, 1998), the effects of salicylic acid, H_2O_2, GSH, GSSG, and ABA were studied. The herbicide primisulfuron was also tested because the ABC transporters mediating the transport of GS conjugates across the tonoplast are strongly induced by herbicides (Tommasini et al., 1997). Cadmium (Cd) has been reported to affect BjOPT1 expression (Bogs et al., 2003) and was therefore also tested. Treatment by salicylic acid (10 μM), GSH (100 μM), GSSG (100 μM), H_2O_2 (5 mM), and Cd (50 μM) did not affect AtOPT6 expression within 5 h (data not shown). A weak increase of expression was observed after 2,4-dichlorophenoxyacetic acid (2,4-D) treatment (Fig. 7). AtOPT6 expression was significantly induced by ABA within 30 min. A strong induction was observed after 30 min of primisulfuron treatment and lasted for at least 90 min (Fig. 7).

Real-time RT-PCR was conducted on various organs of hydroponically grown Arabidopsis plants treated either by cadmium or primisulfuron. Both compounds

were added in the liquid medium 36 h before the plants were collected. Cadmium treatment did not affect *AtOPT6* expression in roots, leaves, and stems (Fig. 8), thus confirming the data obtained with suspension cells. By contrast, primisulfuron almost doubled the expression of *AtOPT6* in roots and stems but had no significant effect in leaves.

Glutathione is known to chelate heavy metals (Perrin and Watt, 1971), and we recently showed that Cd inhibited the expression of BjGT1 (Bogs et al., 2003). This led us to investigate possible interference between AtOPTs, glutathione, and heavy metals by studying yeast growth under different conditions (Fig. 9). In those experiments, all media contained cadmium, and the effects of glutathione addition, and of the presence of either pDR/*AtOPT6* or pDR/*AtOPT7* in the yeast, were studied. The addition of GSH in the medium accelerated the growth of the wild-type yeast, suggesting that glutathione conferred some protection against Cd for this strain (Fig. 9A). In the absence of GSH, the growth curve of the yeast mutant *hgt1* was very similar to that of the wild type in the same conditions. However, in contrast to what was observed for the wild type, the addition of GSH did not affect the growth of the *hgt1* yeast mutant. This was expected since this mutant is affected in glutathione uptake (Bourbouloux et al., 2000). Surprisingly, complementation of the yeast *hgt1* mutant by *AtOPT6* increased its sensitivity to Cd, since the growth curve was delayed compared to the mutant, and this sensitivity was even more increased when GSH was added
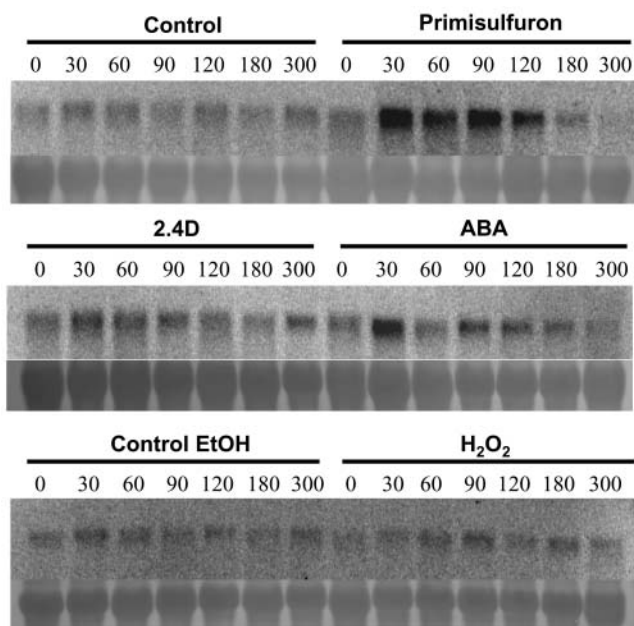
Figure 7. RNA blot analysis of *AtOPT6* expression in Arabidopsis suspension cells treated with primisulfuron (80 nM), 2,4-D (10 μM), ABA (10 μM), ethanol (0.07%), and H_2O_2 (5 mM). The numbers refer to the duration of treatment expressed in min. Ethanol was used as a control for primisulfuron, which was added from an ethanolic stock solution. All treatments were repeated twice independently with similar results.

to the medium (Fig. 9A). Although complementation by *AtOPT7* increased the sensitivity of the mutant to Cd, as shown by a delay in the growth curve, GSH addition did not affect the growth of the *AtOPT7* complemented strain (Fig. 9B), unlike what was observed after *AtOPT6* complementation (Fig. 9A).

DISCUSSION

The importance of peptide transport in plants and the small information available on this process have been recently underlined (Stacey et al., 2002a). The OPT family in Arabidopsis has been described as an oligopeptide transporter family (Koh et al., 2002). Peptide growth assays demonstrated that AtOPT1-7 were able to mediate the uptake of Leu-containing peptides in yeasts and thus complemented Leu auxotrophy (Koh et al., 2002). Furthermore, AtOPT4 was shown to mediate the uptake of KLG-[³H]L, but at a much lower rate than that mediated by CaOPT1 from *Candida albicans*, so that KLGL appeared to be a poor substrate. The same paper mentioned that studies using [³H]glutathione failed to reveal any uptake when yeast cells were expressing any of the AtOPTs. By contrast, two plant homologs of yeast OPT1 have been reported to transport glutathione conjugates, GSSG, and to a lesser extent GSH. Glutathione transport was thus characterized for BjGT1 (Bogs et al., 2003) and OsGT1 (Zhang et al., 2004). This study, therefore, investigates the possibility that members of

the AtOPT family may mediate glutathione uptake, and characterizes the localization of their expression and their inducibility.

AtOPT6 and *AtOPT7* were cloned by PCR and introduced in the yeast mutants ABC 817 (*hgt1met15*) and ABC 822 (*hgt1*) by a 2-μ high copy number vector. Unlike the yeast glutathione transporter HGT1, none of the plant homologs tested (AtOPT6, AtOPT7, OsGT1, BjGT1) was able to restore growth of the *hgt1met15* mutant on a medium containing GSH and sulfate. By contrast, complementation by *AtOPT6*, but not by *AtOPT7*, was able to restore growth of the *hgt1* mutant and to mediate uptake of [³H]GSH in a sulfur-free minimal medium (Figs. 1 and 2). This indicates that the ability of the plant genes to complement glutathione auxotrophy is sufficient only in a genetic background where the need for reduced sulfur compounds is not too strong.

In contrast with AtOPT7, expression of AtOPT6 in the ABC 822 *hgt1* yeast strain restored its ability to grow on a sulfur-free minimal medium containing either GSH or GSSG as the sole sulfur source (Fig. 1). In agreement with a previous report (Koh et al., 2002), this suggests that AtOPT7 is unable to transport GSH and GSSG at a substantial rate. This conclusion is further supported by the fact that the sensitivity of *AtOPT7* yeast transformants to Cd is not enhanced by the presence of GSH (Fig. 9, and see discussion below).

The ability of the ABC 822/pDR/*AtOPT6* yeast to absorb GSH was confirmed by direct uptake measurements under the same conditions (Fig. 2). The characteristics of [³H]GSH uptake mediated by AtOPT6 expression in the yeast were essentially the same as those described previously for BjGT1 and OsGT1. The initial rate of uptake mediated by AtOPT6 was about 0.3 nmol GSH mg^{-1} protein min^{-1}, compared to about 1 nmol GSH mg^{-1} protein min^{-1} for BjGT1 (Bogs et al., 2003) and OsGT1 (Zhang et al., 2004). The initial rate of GSH uptake mediated by AtOPT6 is thus the lowest
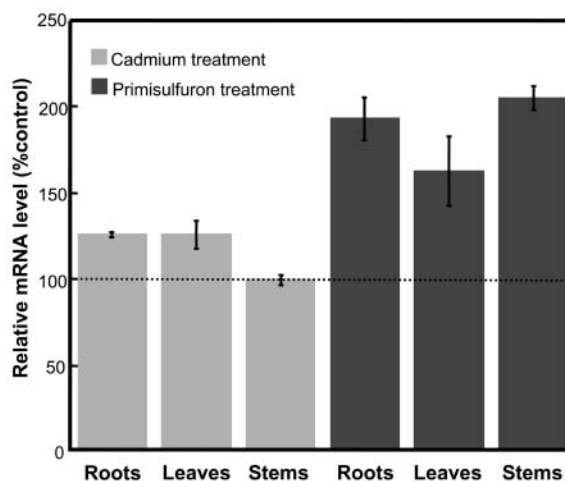
Figure 8. RT-PCR analysis of *AtOPT6* expression in Arabidopsis plants treated with cadmium (50 μM) or primisulfuron (80 nM). The data are means of three measurements ±SE.
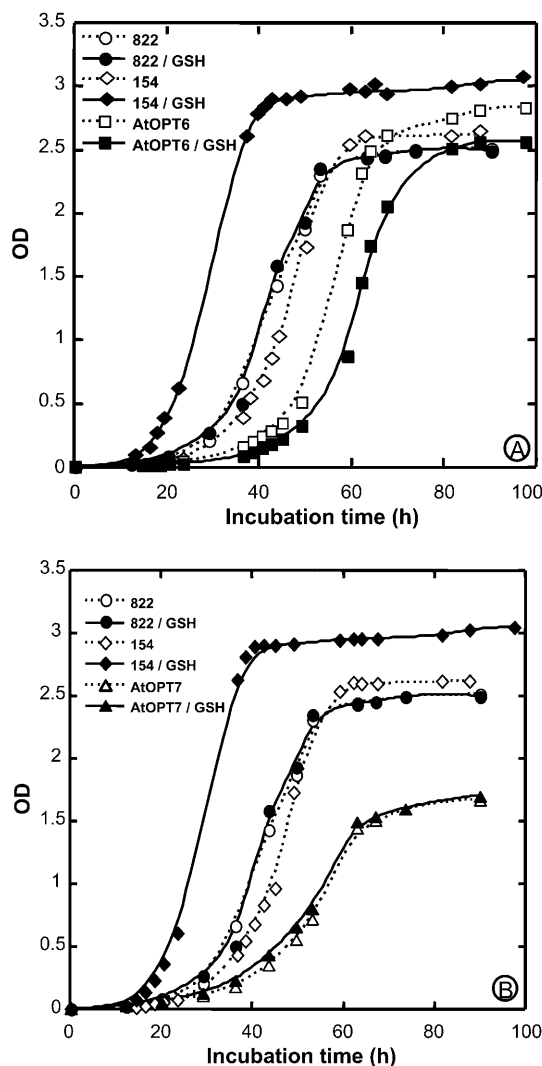
Figure 9. Growth assays of various yeast strains in media containing 50 μM cadmium. A, Effect of transformation by *AtOPT6* and/or GSH addition; 154 is the wild-type strain; 822 is a *hgt1* mutant strain; *AtOPT6* refers to the 822 strain carrying the pDR/*AtOPT6* plasmid. GSH indicates that GSH was present at a final concentration of 50 μM in the medium. B, Effect of transformation by *AtOPT7* and/or GSH addition. All experiments were repeated three times with similar results.

among the plant OPT transporters characterized so far and is about 6-fold less than the uptake rate mediated by the yeast transporter HGT1. As BjGT1 (Bogs et al., 2003) and OsGT1 (Zhang et al., 2004) but unlike HGT1 (Bourbouloux et al., 2000), AtOPT6 mediates GSH uptake in a way that does not obey simple kinetics. Eadie-Hofstee plots yielded a high affinity phase with a K_m of about 400 μM, and a low affinity phase with a K_m of about 5 mM. As discussed earlier (Zhang et al., 2004), it is difficult to know whether the low uptake rate and the biphasic kinetics of the plant transporters actually reflect their intrinsic properties or are due to improper or insufficient targeting of the plant transporters to the yeast plasma membrane. It is also noteworthy that

GSH uptake by isolated wheat (*Triticum aestivum*) chloroplasts also obeyed biphasic kinetics with a high affinity phase (K_m = 30−50 μM) and a low affinity phase with a K_m in the mM range (Noctor et al., 2002).

The substrate specificity of AtOPT6 was studied indirectly by measurements of [³H]GSH uptake in the presence of an excess of potential competitors. An excess of unlabeled GSH inhibited uptake of [³H]GSH only moderately (about 30%) due to the fact that GSH transport may be mediated by the low affinity phase and thus is not completely saturated at 5 mM. In spite of this limitation, the main features observed with OsGT1 (Zhang et al., 2004) were also found for AtOPT6, i.e. a strong competition by GSSG, glutathione-*N*-ethyl-maleimide conjugate, L-Met, L-Gln, and KLLLG. For both transporters, Leu-enkephalin did not compete with GSH uptake, whereas KLLG was a strong competitor. In addition, we show that KLGL (not studied previously) is not a good competitor of GSH uptake mediated by AtOPT6. The main differences derived from competition experiments are that L-Glu is a good competitor for OsGT1 but not for AtOPT6. By contrast, Leu-Leu is a strong inhibitor of GSH uptake mediated by AtOPT6, whereas it has little effect on OsGT1-mediated GSH uptake.

Both *AtOPT6* and *AtOPT7* expression in the ABC 822 (*hgt1Δ*) strain increased its sensitivity to Cd. This result is in agreement with a recent report showing that an OPT family member, AtOPT3, is regulated by metals and that its heterologous expression may rescue yeast mutants deficient in metal transports (Wintz et al., 2003). Furthermore, the AtOPT6-induced Cd sensitivity of the yeast is increased in the presence of GSH, whereas GSH does not affect AtOPT7-induced Cd sensitivity (Fig. 9). These results suggest that AtOPT6, unlike AtOPT7, is able to transport GSH-Cd complexes, in addition to GS conjugates. The effect of GSH on Cd sensitivity in yeasts expressing *AtOPT6* may occur as follows: extracellular complexation of Cd by GSH, uptake of the complex by AtOPT6, release of Cd inside the cells. Whereas the amount of BjGT1 protein in *B. juncea* leaves is decreased by a 96-h exposure of the root system to Cd (Bogs et al., 2003), and whereas the amounts of *AtOPT2* and *AtOPT3* transcripts were strongly enhanced by iron deficiency (Wintz et al., 2003), we failed to detect any effect of Cd treatment of the level of *AtOPT6* transcripts analyzed by quantitative RT-PCR. It is difficult to conclude to a general pattern of OPT regulation by metals because these data concern different members of the OPT family, and because there is not always a univocal correlation between the transcripts and protein amounts of OPTs, suggesting that this family may be subject both to transcriptional and translational regulations (Bogs et al., 2003; Zhang et al., 2004). Nevertheless, there are now several lines of evidence that at least some of the members may be able to transport metals and/or metal-peptide complexes. The expression of either *AtOPT6* or *AtOPT7* increases the sensitivity to Cd, even in the absence of GSH, compared to

the mutant strain (Fig. 9). This suggests that both transporters may transport either Cd or, more likely, a complex between Cd and a component of the growth medium (His, for example).

Koh et al. (2002) studied the expression of various *AtOPT*s by RT-PCR. They found that both *AtOPT6* and *AtOPT7* were expressed in the flowers and, to a lower extent, in the roots. The data in this study extend these observations by a detailed analysis of GUS expression driven by either the *AtOPT6* or *AtOPT7* promoters. Thus, we show that in the flowers, the expression is mainly restricted to the filaments of the stamen and to the vascular bundles irrigating the young seeds. In the roots, *AtOPT6* was mostly expressed in the regions of lateral root initiation. In this respect, it is interesting to note that the *cad1* mutant blocked in glutathione synthesis is also impaired in the activation of cell division in root meristem (Vernoux et al., 2000). Furthermore, *AtOPT6*-driven GUS expression was also observed in the cambial zone of petiole and stem vascular bundles. The pattern of expression observed in the roots, stems, and petioles thus suggests that AtOPT6 is mainly expressed in areas where cell division is active. The pattern of AtOPT7 expression markedly differs from that of AtOPT6, in that it was poorly expressed in the conducting bundles (Fig. 6). This different pattern of expression, the different phenotypes conferred by AtOPT6 and AtOPT7 related to glutathione uptake, and Cd sensitivity in yeasts clearly indicate that these two transporters, in spite of their strong homology, exert different functions in the plant.

The metabolism of both primisulfuron and ABA is mediated via glucosylation. It is thus unexpected that both compounds induce the expression of a transporter so far characterized as a glutathione transporter. However, the same situation has been described for the induction of ABC transporters of the multidrug resistance proteins family by xenobiotics. Tommasini et al. (1997) studied the effects of primisulfuron and other biotics on the expression of four Arabidopsis ABC transporters homologous to MRPs. The abundance of one transcript called EST2 was strongly increased by primisulfuron and 1-chloro-2,4-dinitrobenzene, which are detoxicated as a Glc and a glutathione conjugate, respectively, but not by Cd (detoxicated by phytochelatins and glutathione complexes) and metolachlor (sequestered as glutathione conjugates). This indicated that plant may respond to toxic compounds with an induction of a large spectrum of proteins that may be related to various aspects of detoxification. The fact that AtOPT6 is strongly induced by primisulfuron and ABA suggests that it may play a role in detoxification and/or stress resistance, but the lack of effect of H_2O_2 indicates that oxidative stress is not directly involved.

In conclusion, these data and those described by Koh et al. (2002) suggest that AtOPT6 may transport peptides, glutathione conjugates, and glutathione complexes. However, as underlined by these authors, it is difficult to ascertain what are the most relevant substrates in vivo and thus the exact function of the AtOPT members. The comparison between AtOPT6 and AtOPT7 clearly indicates that the glutathione transport capacity is not shared by all the members of the family. Induction of AtOPT6 by primisulfuron and ABA suggests that this transporter may be involved in detoxification process.

MATERIALS AND METHODS

Cloning of *AtOPT6* and *AtOPT7* cDNAs

The PCR experiments were performed using cDNA prepared from 21-d-old plants and the Pfu turbo polymerase (Promega, Madison, WI). The *AtOPT6* cDNA (GenBank accession no. AL035602.1) was cloned by PCR using primers *AtOPT6-forward* (5′CTCTTCTAAAACGATGGGAGAG3′) and *AtOPT6-reverse* (5′GTACAAAGCACTTGGTATATGC3′). The *AtOPT7* (BAC clone T12H20, GenBank accession no. AF080119.1) coding sequence was cloned by PCR using primers *AtOPT7-forward* (5′GAAGACAACCATVGGAAGAATC3′) and *AtOPT7-reverse* (5′GGTACCAAATCCAACCACC3′). The PCR products were cloned into the pGEM-T Easy (Promega) vector and entirely sequenced.

Plasmid Constructs For Expression in Yeast

The 2.27-kb and 2.37-kb PCR fragments corresponding, respectively, to the open reading frame of *AtOPT6* and *AtOPT7* cDNAs were subcloned from the pGEM-T Easy vector into the *Eco*RI site of yeast expression vector pDR195 (*AtOPT6*) and into the *Not*I site of pDR196 (*AtOPT7*). The vectors pDR195 and pDR196 were kind gifts of Doris Rentsch (University of Bern, Switzerland).

Yeast Growth

The *Saccharomyces cerevisiae* strain ABC 822 bearing a deletion in *HGT1* (*MATa ura3-52 leu2-Δ1 lys2-801 his3-Δ200 trp1-Δ63 ade2-101 hgt1Δ::LEU2*) and ABC 817 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 hgt1Δ::LEU2*) yeasts (Bourbouloux et al., 2000) are deficient in glutathione uptake and were used for complementation studies.

SD-S medium was prepared according to DIFCO's Bacto yeast nitrogen base without amino acids and ammonium sulfate recipe (DIFCO Laboratories, Detroit), with the modification that all sulfur containing reagents were substituted with equal amounts of the corresponding chloride salt (Zhang et al., 2004). The *S. cerevisiae* strain ABC 822 and the strain ABC 822 transformed with empty vectors (ABC 822/pDR) were grown in DIFCO's SD medium with ammonium sulfate, or in synthetic complete medium (Sherman, 1991), which contains ammonium sulfate and sulfur amino acids.

Transport Experiments

For growth assays in liquid and on solid medium, cells of ABC 822/pDR and ABC 822/pDR/AtOPT were grown overnight to an $OD_{600} = 0.6$ in minimal liquid SD medium containing ammonium sulfate and 2% Glc and the necessary amino acids. Uptake experiments were conducted as described by Zhang et al. (2004). Briefly, after a 5-min incubation of the cells at 28°C, [³H]GSH (1.9 TBq mmol⁻¹; Amersham France, Les Ulis, France) was added to the buffered transport medium to a final concentration of 0.1 mM GSH (final specific activity 38 MBq mmol⁻¹). At selected times, uptake was stopped by diluting the medium with a 20-fold volume of water (4°C), and cells were separated from the medium by filtration through a glass fiber filter (Sartorius AG, Goettingen, Germany). The cells were washed, the filter was dried, and the radioactivity was counted as described (Zhang et al., 2004).

Growth Test Experiments

The yeast were grown in yeast nitrogen base (DIFCO Laboratories) supplemented with ammonium sulfate, 2% Glc, and necessary auxotrophic supplements until an OD_{600} of 0.6. Twenty-five milliliters of fresh medium were inoculated to reach an initial OD_{600} of 9.7×10^{-3}. The yeast were grown on a rotary shaker (200 rpm) at 28°C, and the OD_{600} was monitored for about

100 h by sampling 500-μL aliquots. In some experiments, $CdCl_2$ (50 μM, final concentration), GSH, or GSSG (500 μM) were added to the medium as described in the "Results" section.

To check for glutathione auxotrophy, the yeasts were grown in SD-S liquid medium supplemented with either 500 μM GSH or GSSG.

Plant Growth

Seeds of Arabidopsis (Columbia ecotype) were grown hydroponically in nonsterile conditions. Cell suspensions of Arabidopsis (ecotype Columbia, strain T87) were cultured as described previously (Axelos et al., 1992).

Induction Experiments

The cultures were kept on a rotary shaker at 120 rpm and under 16-h photoperiod. Stress treatments were conducted 6 d after inoculation. Phytohormones (ABA) and growth regulators (2,4-D) were added at 10 μM (final concentration). The herbicide primisulfuron (80 nM), $CdCl_2$ (50 μM), and hydrogen peroxide (5 mM) were directly administered into the culture medium. At the end of the treatment, plant cells were washed twice with deionized water and deep-frozen before RNA extraction.

In another set of experiments, Arabidopsis plants were grown hydroponically. Primisulfuron (80 nM) and $CdCl_2$ (50 μM) were added to the liquid medium 36 h before the plants (3 weeks old) were sampled.

RNA Isolation and RNA Blot

Total RNA was isolated from yeasts as described by Logemann et al. (1987). Northern blots were performed following standard protocols (Sambrook et al., 1989), with 10 μg of total RNA in each lane. The full-length *AtOPT6* cDNA was labeled by random primer labeling with ^{32}P-dCTP and used as a probe.

Quantification of AtOPT Transcripts by Real-Time PCR

Real-time PCR-specific primers to AtOPT6 (*forward*, GAGTAGAACT-CAATTCTTCTTAA; *reverse*, ACCAGAATTGATTTGGGATTGA) and to eIF1α (*forward*, TGTAACAAGATGCATGGCACT; *reverse*, ATGGGCACAAATGG-GATTTT) were designed in the coding sequence of AtOPT6 and eIF1α. The specificity of the PCR product was checked by sequencing. For real-time PCR, total RNA was isolated from leaves, stems, and roots of 5-week-old hydroponically grown Arabidopsis plants. Either the herbicide primisulfuron or $CdCl_2$ was added to the growth solution at final concentrations of 80 nM and 50 μM, respectively, 36 h before harvesting the plants. Five micrograms of the total RNA were reverse transcribed with a cDNA synthesis kit (MMLV reverse transcriptase; Gibco-BRL, Cleveland). Quantitative real-time PCR was carried out using an ABI Prism 7700 cycler (Applied Biosystems, Foster City, CA) and the SYBR Green PCR master mix (Applied Biosystems).

Histochemical GUS Assays

The promoter regions of *AtOPT6* and *AtOPT7* were amplified by PCR from genomic DNA. Genomic DNA was prepared from whole plants using the Wizard kit (Promega). The primers used were: *ProAtOPT6-forward*, ATCCGTGTGGTCATTATTTAGC, and *ProAtOPT6-reverse*, GAGTGAGTGT-TCCGTTCTTTG; *ProAtOPT7-forward*, TGTTTTCTT TCCTACCACTGG, and *AtOPT7-reverse*, CTTCTTCTTCTTCTTGCTCTG. Amplification allowed to recover a 2.4-kb and a 2.0-kb fragment of the AtOPT6 and AtOPT7 promoter regions, respectively. These fragments were subcloned into the pSK vector and sequenced. They were subcloned into the *Xba*I/*Sma*I cloning site of the pCB308 vector containing the *uidA* reporter gene sequence (Xiang et al., 1999). The constructs were then introduced into *Agrobacterium tumefaciens* strain EHA105 by electroporation and selected for resistance to kanamycin (50 μg/mL). Arabidopsis ecotype Columbia was transformed by vacuum infiltration, and seeds were selected by spraying with glufosinate ammonium (BASTA, Frankfurt). Individual transformed plants were selfed to select for homozygous plants. At least five independent transformants were analyzed for each construct. One- to 3-week-old plants were stained by 5-bromo-4-chloro-3-indolyl-β-GlcUA by standard procedures. Samples were observed and photographed using a light microscope.

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