Arabidopsis OPT6 is an Oligopeptide Transporter with Exceptionally Broad Substrate Specificity

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Abstract: Oligopeptide transporters (OPTs) are found in fungi, bacteria and plants. The nine Arabidopsis thaliana OPT genes are expressed mainly in the vasculature and are thought to transport tetra- and pentapeptides, and peptide-like substrates such as glutathione. Expression of AtOPT6 in Xenopus laevis oocytes demonstrated that AtOPT6 transports many tetra- and pentapeptides. In addition, AtOPT6 transported reduced glutathione (GSH), a tripeptide, but not oxidized glutathione (GSSG). Recent data showed that Candida albicans OPTs can transport peptides up to eight amino acids in length. AtOPT6 transported mammalian signaling peptides up to 10 amino acids in length and, in addition, known plant development- and nematode pathogenesis-associated peptides up to 13 amino acids long. AtOPT6 displayed high affinity for penta- and dodecapeptides, but low affinity for GSH. In comparison the Saccharomyces cerevisiae ScOPT1 was incapable of transporting any of the longer peptides tested. These data demonstrate the necessity of experimentally determining substrate specificity of individual OPTs, and lay a foundation for structure/function studies. Characterization of the AtOPT6 substrate range provides a basis for investigating the possible physiological function of AtOPT6 in peptide signaling and thiol transport in response to stress.

Keywords: Arabidopsis • CLE peptides • Membrane transporter • Oocytes • Two-electrode voltage clamp • Xenopus laevis.

Abbreviations: ABC, ATP-binding cassette; CLE, CLAVATA3/ ENDOSPERM SURROUNDING REGION; γEC, γ-Glu-Cys; GNOS, S-nitrosoglutathione; GSH, reduced glutathione; GSSG, oxidized glutathione; GUS, β-glucuronidase; I_{max}, maximal current; K_{0.5}, substrate concentration at half-maximal current; OPT, oligopeptide transporter; PC₂ (γ-Glu-Cys)₂-Gly (phytochelatin); UTR, untranslated region.

Introduction

Transport of peptides across cell membranes is well known in bacteria, fungi, plants and animals (Stacey et al. 2002a). In plants, three transmembrane protein families were identified that transport peptides. Members of the ATP-binding cassette family (ABC) transport diverse substrates (Blackmore et al. 2001). The peptide transporter family, also named the proton oligopeptide transporter (POT) family, was shown to transport di- and tripeptides, nitrate and amino acids (Williams and Miller 2001, Stacey et al. 2002a, Waterworth and Bray 2006, Tsay et al. 2007). Oligopeptide transporters (OPTs) have been thought to transport the tripeptide glutathione, or peptides 4–5 amino acids in length (Lubkowitz et al. 1997, Bourbouloux et al. 2000, Stacey et al. 2002a, Waterworth and Bray 2006). First found in Candida albicans (Lubkowitz et al. 1997), OPTs have been identified by database comparisons in bacteria (Yen et al. 2001), other fungi (Lubkowitz et al. 1998) and plants, including rice (Zhang et al. 2004), Brassica juncea (Bogs et al. 2003) and Arabidopsis thaliana (Koh et al. 2002).

It is not clear what physiological functions OPTs serve in multicellular organisms or how the peptide-transporting systems interact with each other. In Saccharomycyes cerevisiae, OPT1 may primarily transport glutathione (Bourbouloux et al. 2000), but it also permits auxotrophic yeast to take up and grow on Leu-enkephalin, a pentapeptide (Hauser et al. 2000, Osawa et al. 2006, Wiles et al. 2006). OPT transport may not be limited to tri-, tetra- and pentapeptides.
Wild-type C. albicans, but not opt triple mutants, utilized peptides up to eight amino acids in length for growth (Reuß and Morschhäuser 2006). In fungi, OPTs may mainly function in scavenging peptides and peptide-like molecules for nutritional purposes.

In Arabidopsis, nine members of the OPT family were identified (Koh et al. 2002). OPT promoter–GUS (β-glucuronidase) expression constructs showed that AtOPT genes are mainly expressed in the vasculature throughout the plant, with minor differences in tissue-specific expression during vegetative growth and larger differences during reproduction (Stacey et al. 2006). None of the Arabidopsis OPT genes is expressed in the root epidermis or root hair cells, thus indicating that unlike in fungi, plant OPTs do not function in nutrient uptake from the environment. They may have overlapping functions, but also play specific roles. Analysis of OPT knock-out lines has not led to phenotypic identification of function or suggestion of physiological substrates, with the exception of AtOPT3 (Stacey et al. 2006). Knock-outs in AtOPT3 are arrested early in embryonic development (Stacey et al. 2002b). More recent data suggest that this phenotype is due to defects in metal homeostasis, implicating AtOPT3 in iron transport (Stacey et al. 2008).

Strong AtOPT6 promoter–GUS expression in the vasculature of root, stem and leaf (Cagnac et al. 2004, Stacey et al. 2006) suggests that it is involved in long-distance peptide transport or distribution throughout the plant. In stems and leaves, GUS reporter expression is strongest in the cambial zone of the vascular bundles (Cagnac et al. 2004). AtOPT6 probably has overlapping functions with other AtOPT genes. For example, AtOPT6, like AtOPT1, AtOPT4, AtOPT7 and AtOPT8, is expressed in the root vascular cylinder (Stacey et al. 2006). AtOPT6 also probably plays specialized roles in growth and reproduction: only AtOPT6 is expressed during lateral root emergence and in unfertilized ovules. After fertilization, AtOPT6 is expressed strongly in the vasculature of the ovary and style (Stacey et al. 2006).

In plants, OPT-mediated peptide transport could serve a number of important functions. Research suggested that glutathione transport is important for sulfur distribution and maintenance of redox potential, a function that controls a host of cellular functions including resistance to heavy metal stress and oxidative stress, growth and development (Noctor et al. 1998, Meyer and Hell 2005, Mullineaux and Rausch 2005, Maughan and Foyer 2006). It has been suggested that OPT transport of other peptides could distribute nitrogen, regulate peptide activity with peptide-conjugated hormones or move peptide signals (Stacey et al. 2002a). Glutathione and peptides were found both in phloem (Kuzuhara et al. 2000, Mendoza-Cózatl et al. 2008) and in xylem sap (Macduff et al. 1994, Escher et al. 2003).

Growth and uptake studies with mutant yeast expressing AtOPT6 produced conflicting data on the transport of glutathione and tetra- or pentapeptides (Koh et al. 2002, Cagnac et al. 2004, Osawa et al. 2006). Here, we used an optimized AtOPT6 expression construct and heterologous expression in Xenopus oocytes to measure peptide transport by AtOPT6. We show that AtOPT6 can transport reduced glutathione (GSH) with low affinity, but oxidized glutathione (GSSG) is not transported. Additionally, AtOPT6 can transport many tetra- and pentapeptides with high affinity.

Surprisingly, we found that AtOPT6 can transport peptides much longer than tetra- and pentapeptides, including peptides at least 12–13 amino acids long such as CLAVATA3-like peptides that play a role in plant development and a root-knot nematode protein that is needed for pathogenesis. Comparisons of AtOPT6 transport characteristics with those of ScOPT1 and AtOPT4 illustrate that presently it is not possible to predict which substrates are transported by OPTs, necessitating experimental determination of the substrate range for individual OPTs. However, marked differences in substrate selectivity between OPTs as shown here lay a foundation for structure/function studies. Characterization of the exceptionally broad AtOPT6 substrate range suggests testable hypotheses regarding in planta AtOPT6 function.

Results

AtOPT6 transports GSH and phytochelatin

In the previous electrophysiological study of OPT transporters, no currents were detected with any tested substrate, including GSH, in measurements of Xenopus oocytes injected with AtOPT6 cRNA (Osawa et al. 2006). Subsequently, we optimized AtOPT6 expression in oocytes by swapping the 5′-untranslated region (UTR) of AtOPT6 with the 5′-UTR of ScOPT1 (see Materials and Methods), as was previously done with AtOPT4 (Osawa et al. 2006). AtOPT6 is not changed in amino acid sequence in this construct. To identify substrates transported by AtOPT6 in oocytes using a two-electrode voltage clamp, we first tested commercially available peptides that had been used in other OPT studies. We were particularly interested in glutathione because the reduced tripeptide GSH or oxidized GSSG occur in plants and are potential endogenous substrates for AtOPTs. We also tested three other GSH-related plant peptides: γ-Glu-Cys (γEC), S-nitrosoglutathione (GNOS) and (γ-Glu-Cys)2-Gly [phytochelatin (PC2)]. GSH and GSSG were both reported to permit the growth of a S. cerevisiae mutant disrupted in the endogenous glutathione transporter ScOPT1/SCHGT1 (Bourbouloux et al. 2000) and transformed with AtOPT6 (Cagnac et al. 2004). However, GSH did not aid the growth of this transformed yeast mutant in another study (Osawa et al. 2006).

Of the GSH-related compounds tested, GSH and PC2 were the only substrates that caused significantly more negative
(‘inward’) currents in AtOPT6-expressing oocytes than in uninjected oocytes, indicating that they are the only ones transported by AtOPT6 (Fig. 1). Inward currents caused by GSH were twice those caused by PC$_2$ in AtOPT6-injected oocytes (Fig. 1). Oocyte expression of heterologous transporters varies depending on oocyte batch and day after injection. In subsequent experiments, we applied 500 µM GSH first for evidence of adequate expression (see Materials and Methods).

**AtOPT6 is a proton-coupled low affinity GSH transporter**

By electrophysiological convention, inward currents signify the uptake of net positive charges or the release of net negative charges. While GSH and PC$_2$ carry net negative charges, the fact that inward currents were induced when external substrate was added to AtOPT6-expressing oocytes indicated that uptake of GSH and PC$_2$ was coupled to positive charges. In plants, the plasma membrane proton pump energizes the plasma membrane, creating both a proton and an electrical gradient across the membrane (Sondergaard et al. 2004). The inside is more negative and has a higher pH, making proton-coupled substrate transport energetically advantageous.

![Fig. 1](https://example.com/fig1.png)

**Fig. 1** AtOPT6 can transport reduced glutathione (GSH) and phytochelatin (PC$_2$), but not several other GSH-related substrates. Each oocyte was exposed to 500 µM GSH and subsequently to one or more GSH-related substrate at 500 µM and pH 5.0, and held at a membrane potential of −80 mV. Results are averages of difference currents (difference in current before and after substrate addition ± SD) for 4–29 AtOPT6-expressing oocytes. GSH (n = 29), PC$_2$ (n = 16), GSSG (n = 5), GNOS (n = 4) and γEC (n = 6). *Significantly different from all other treatments at the 0.05 level; ns, not significantly different from uninjected control oocytes. In uninjected control oocytes there were no significant differences between substrates (n = 3–14 oocytes per substrate).

![Fig. 2](https://example.com/fig2.png)

**Fig. 2** AtOPT6 currents are pH dependent and saturable. (A) pH dependence of AtOPT6 transport. Current resulting from 1 mM GSH was measured at a holding potential of −80 mV and pH 5, pH 6, and pH 7 for each oocyte. Results are average difference currents ± SD at each pH. The slope of the line for uninjected controls is not significantly different from 0 at the 0.05 level. For AtOPT6-expressing oocytes the difference is significant (P < 0.0001). (B) Currents elicited by GSH in AtOPT6-expressing oocytes saturate between 1.25 and 5 mM. Representative current–voltage relationships for different concentrations of GSH at pH 5.0. A corresponding uninjected oocyte response is shown for comparison. The membrane potential was clamped between +20 and −80 mV in −20 mV increments at each concentration.

Oocytes expressing AtOPT6 exhibited significantly more inward current from GSH application at pH 5 than at pH 6 or pH 7 (Fig. 2A), an indication that AtOPT6 transport is proton coupled. To determine the affinity of AtOPT6 for GSH, we measured currents at −80 mV with increasing GSH concentrations, from which we calculated maximal current ($I_{max}$) and the substrate concentration at which currents are half-maximal ($K_{0.5}$). Glutathione-evoked currents were saturable between 1.25 and 5 mM (Fig. 2B) with a $K_{0.5}$ of >500 µM (Table 1). Thus, AtOPT6 is a low-affinity GSH transporter. In contrast, 5 mM PC$_2$ did not saturate the current (data not shown), indicating that the affinity of AtOPT6 for PC$_2$ is even lower than for GSH.

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**References**


**Table 1**

<table>
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<tr>
<th>Substrate</th>
<th>$n$</th>
<th>$K_{0.5}$ (µM)</th>
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<tr>
<td>GSH</td>
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<td>PC$_2$</td>
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<td>γEC</td>
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OPT6 also transports tetrapeptides

In addition to glutathione, we tested tetra- and pentapeptides that were previously used in studies with yeast expressing AtOPT6 (Koh et al. 2002, Cagnac et al. 2004, Osawa et al. 2006) or with Xenopus oocytes expressing ScOPT1 and AtOPT4 (Osawa et al. 2006). Although these peptides are not known to occur in plants, they either permitted growth of auxotrophic yeast expressing AtOPT6 (Koh et al. 2002) or were transported by Xenopus oocytes expressing ScOPT1 or AtOPT4 (Osawa et al. 2006). Large inward currents were seen in AtOPT6-expressing oocytes when 500 µM GGFL, GGFM or KLGL were applied (Fig. 3). In the same oocyte, these peptides caused currents that were 2–6 times the GSH-induced currents, suggesting a higher affinity or transport rate of AtOPT6 for these peptides. Similar to GSH, GGFL-induced currents were pH dependent (data not shown). While GSH carries a net negative charge, the net charge of GGFL is zero. For ScOPT1, it was shown that the apparent affinity of the transporter for protons was fairly constant, with substrate charges ranging from 0 (GGFL) to –2 (GSSG) (Osawa et al. 2006).

Both amino acid sequence and peptide length affect transport by AtOPT6

Very little is known about the substrate characteristics that determine permeability through an OPT transporter. We examined the influence of peptide length and amino acid sequence on AtOPT6 inward currents using a series of peptides from KG to KLLLLG, including the inverse pentapeptide, GLLLK. As expected, the dipeptide KG did not induce greater inward currents in AtOPT6-injected oocytes than in uninjected oocytes (Fig. 4A). Application of the tripeptide KLG caused small but significant currents (Fig. 4A, B). Consistent with the idea that AtOPT6 is a tetra- and pentapeptide transporter, inward currents caused by KLG and KLLLLG application were 3- to 7-fold greater than with KLG (Fig. 4B).

Further examination of the current kinetics of the pentapeptide showed that at −80 mV the $K_{0.5}$ for KLLLLG was approximately 10 µM (Table 1), similar to the high affinity transport of KLG by AtOPT4 (Osawa et al. 2006). These data indicate that AtOPT6 can transport pentapeptides with high affinity. However, the inverse pentapeptide, GLLLK, induced only a small inward current in AtOPT6-expressing oocytes similar to that caused by KLG (Fig. 4A), indicating that peptide amino acid sequence, rather than content, contributes to the substrate selectivity of AtOPT6. Also, the large inward currents evoked by the hexapeptide KLLLLG (Fig. 4A, B) indicated that AtOPT6 transports peptides longer than five amino acids, probably also with high affinity.

We therefore tested the bradykinin pentapeptide RPPGF and heptapeptide RPPGFSP, and the angiotensin octapeptide DRVYIHPFHL, all of which permitted growth of C. albicans when supplied as the sole nitrogen source (Reuß and Morschhäuser 2006). We further tested related longer peptides, the nona- and decapeptides RPPGFSPFR and DRVYIHPFHL. These are signaling peptides in mammals but are not known to occur in plants. Large currents were induced in AtOPT6-expressing oocytes by these peptides and there were no significant differences among them (Fig. 5), indicating that these pentapeptides and longer peptides are transported with similar efficiency.

OPT6 can transport plant signaling peptides

Of the above tested peptides, only GSH, its precursor γEC, and its derivatives such as PC, are known to occur in plants. However, the high affinity of AtOPT6 for peptide substrates and the surprising variability in peptide size of transported substrates prompted us to test peptides known to perform

Table 1 Apparent affinities of AtOPT6 for peptide substrates

<table>
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<th>Substrate</th>
<th>$K_{0.5}$ (µM)</th>
<th>SEM</th>
<th>n</th>
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<tr>
<td>GSH</td>
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<tr>
<td>KLLLLG</td>
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<tr>
<td>AtCLE19p12</td>
<td>71</td>
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Inward currents obtained from AtOPT6-expressing oocytes exposed to substrates at pH 5.0 and a holding potential of −80 mV were plotted against substrate concentrations. $K_{0.5}$ values were determined by fitting the Michaelis–Menten equation to the curves. Values represent the means ± SEM for 5–6 oocytes.

Fig. 3 AtOPT6 transports tetrapeptides. Oocytes were exposed to 500 µM GGFL, GGFM and KLGL peptides at pH 5.0 and a holding potential of −80 mV. Results are averages of difference currents ± SD. Currents elicited by GGFL, GGFM and KLGL in AtOPT6-expressing oocytes ($n = 19, 5,$ and $6,$ respectively) are significantly different from those in uninjected oocytes ($n = 3–9 per substrate$), but are not different from each other ($P < 0.05$).

Fig. 4A, B Consistent with the idea that AtOPT6 is a tetra- and pentapeptide transporter, inward currents caused by KLG and KLLLLG application were 3- to 7-fold greater than with KLG. Further examination of the current kinetics of the pentapeptide showed that at −80 mV the $K_{0.5}$ for KLLLLG was approximately 10 µM (Table 1), similar to the high affinity transport of KLG by AtOPT4 (Osawa et al. 2006). These data indicate that AtOPT6 can transport pentapeptides with high affinity. However, the inverse pentapeptide, GLLLK, induced only a small inward current in AtOPT6-expressing oocytes similar to that caused by KLG (Fig. 4A), indicating that peptide amino acid sequence, rather than content, contributes to the substrate selectivity of AtOPT6. Also, the large inward currents evoked by the hexapeptide KLLLLG (Fig. 4A, B) indicated that AtOPT6 transports peptides longer than five amino acids, probably also with high affinity.

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OPT6 can transport plant signaling peptides

Of the above tested peptides, only GSH, its precursor γEC, and its derivatives such as PC, are known to occur in plants. However, the high affinity of AtOPT6 for peptide substrates and the surprising variability in peptide size of transported substrates prompted us to test peptides known to perform
significant signaling roles in plants as well. These included the amino acid conjugate jasmonate–isoleucine, the active form of the oxylipin signal jasmonic acid (Staswick and Tiryaki 2004), and the sulfated pentapeptide hormone phytosulfokine (Matsubayashi and Sakagami 1996). Neither of these substrates induced detectable currents in AtOPT6-expressing oocytes (data not shown).

Some members of the CLAVATA3/ENDOSPERM SURROUNDING REGION (CLE) signaling peptides are known to be signals that control cell differentiation and plant development (Fiers et al. 2007). The active form of CLV3 is 12 (Kondo et al. 2006) or 13 (Ohyma et al. 2009) amino acids in length. CLE-like peptides are also secreted by plant pathogenic nematodes and may function in host cell reprogramming (Mitchum et al. 2008). We tested AtCLE5 (RVSPGGPDQHH), AtCLE19 (RVIPGTPNPPLHN) and HgCLE (RLSPSGPDPHHHH) from *Heterodera glycines* (soybean cyst nematode) and MI16D10p13 (GKKPSGPNPGGN) from *Meloidogyne incognita* (root-knot nematode).

In AtOPT6-expressing oocytes, solutions containing 500 µM CLE and CLE-like peptides induced inward currents (*Fig. 6*) that were in the range of PC₂-induced currents (*Fig. 1*) and were significantly different from those in uninjected oocytes. Furthermore, currents induced by plant and nematode CLEs did not differ significantly from each other (*Fig. 6*), indicating that pathogen signaling peptides could utilize plant OPT transporters to gain entry into host cells. We further characterized the current caused by AtCLE19p12 at a holding potential of −80 mV. The $K_{0.5}$ of AtOPT6 for AtCLE19p12 was about 70 µM (*Table 1*). These data indicate that unlike PC₂, CLE peptides are transported by AtOPT6 with high affinity, and the currents, although small, could be physiologically significant.
Transport properties of AtOPT6 and ScOPT1 are distinct

In marked contrast to AtOPT6-expressing oocytes, 500 µM KLLLG, RPPGF, DRVYIHPF or AtCLE19p12 caused no inward currents in ScOPT1-expressing oocytes greater than those in uninjected oocytes (Fig. 7). We used GSH to show that these oocytes are expressing ScOPT1 (Fig. 7). As is commonly observed with transporter expression in oocytes, ScOPT1 expression was highly variable from batch to batch. Average ScOPT1-mediated currents ranged from −23 ± 4.24 nA (n = 2) in the lowest expressing batch to −181 ± 74.95 nA (n = 2) in the highest expressing batch. Nevertheless, average ScOPT1-mediated GSH currents were significantly different from uninjected oocytes within batches and when data from all five batches were averaged (Fig. 7). We confirmed that ScOPT1 also transports PC2, GSSG and GGFL, but not KLGL, a different pattern from AtOPT6 (Osawa et al. 2006). In contrast to the low affinity GSH transport of AtOPT6 (Table 1), ScOPT1 is a high affinity GSH transporter (Osawa et al. 2006). Thus, AtOPT6 transport function is very distinct from that of ScOPT1.

Discussion

In this report, we determined the substrate range of AtOPT6 by measuring peptide currents in *Xenopus* oocytes using the very sensitive two-electrode voltage clamp technique. Although we cannot formally exclude that AtOPT6 functions as a ligand-gated ion channel with peptides being ligands, it is very likely that the currents measured here represent proton-coupled peptide transport because AtOPT6 was shown to rescue growth of a leucine auxotrophic yeast strain with KLLLG as the sole leucine source (Koh et al. 2002) and enabled uptake of radiolabeled GSH in a yeast GSH uptake mutant (Cagnac et al. 2004). This conclusion is further supported by the positive shifts in current–voltage curves with increasing external substrate concentrations. Here, we showed that AtOPT6 is both a low affinity transporter for GSH and a high affinity transporter for pentapeptides and longer peptides. Unlike GSH, most peptides used in this study are not known to occur in planta. We suggest that glutathione transport may be an important function of AtOPT6 that may be shared by other plant OPTs. The reduced sulfate-containing organic compound GSH is mainly synthesized in leaves. However, it is found throughout the plant where it is thought to play numerous roles in plant development and resistance to stress and pathogens (Noctor et al. 1998, Meyer and Hell 2005, Mullineaux and Rausch 2005, ...
Parisy et al. 2007). Long-distance transport of GSH as the major thiol-containing compound has been well documented (Rennenberg et al. 1979, Lappartient and Touraine 1996, Kuzuhara et al. 2000, Mendoza-Cózatl et al. 2008).

A low affinity transporter could function in GSH long-distance movement because GSH concentrations as high as 200 µM (Mendoza-Cózatl et al. 2008) and 4–5 mM (Kuzuhara et al. 2000) were measured in phloem sap of *Brassica napus* and rice, respectively. AtOPT6 is a prime candidate for the transporter that would be needed for uptake of GSH into phloem companion cells or to permit uptake of transported GSH into cells in other parts of the plant. Promoter–GUS experiments showed that AtOPT6 is expressed throughout the vasculature of *Arabidopsis* seedlings (Cagnac et al. 2004, Stacey et al. 2006). In adult plants, it is expressed in the cambial zone of petiole and stem vascular bundles and in the vasculature of leaf and ovary (Cagnac et al. 2004, Stacey et al. 2006). Further experiments localizing AtOPT6 to the plasma membrane of specific cell types are needed to support a function for AtOPT6 in long-distance movement of GSH.

Our measurement showing that GSH is transported with low affinity by AtOPT6 contrasts with the high affinity GSH transport by ScOPT1 (Osawa et al. 2006) and explains some of the variability in reports of growth and uptake studies with auxotrophic *S. cerevisiae* expressing these transporters (Koh et al. 2002, Cagnac et al. 2004, Osawa et al. 2006). AtOPT6 has almost a 10-fold lower affinity for GSH than ScOPT1. With 100 µM GSH in the medium, marginal uptake of GSH by yeast expressing AtOPT6 could permit moderate growth, whereas high affinity uptake of GSH could cause toxic levels to those expressing ScOPT1 (Osawa et al. 2006). Concentrations of GSH used in other reports of mutant yeasts expressing AtOPT6 ranged from 50 µM [3H]GSH to 500 µM (Koh et al. 2002, Cagnac et al. 2004), all below the average $K_{0.5}$ for AtOPT6 in our system. Therefore, the expression levels in yeast of heterologous AtOPT6 under specific growth conditions may lead to variable results.

A different factor probably accounts for the discrepancy between our results showing that GSSG, the oxidized form of glutathione, is not transported by AtOPT6, and the report that GSSG was a very good competitor for labeled GSH uptake by AtOPT6-expressing mutant yeast (Cagnac et al. 2004). Because GSSG consists of two GSH molecules joined by a disulfide bond, it is not a linear molecule and thus is more bulky. It is possible that GSSG appeared to be a very good competitor because such yeast uptake competition studies do not discriminate between transported substrates and substrates that block the transporter (Rentsch et al. 1998). The observations that 500 µM GSH and GSSG both permitted AtOPT6-expressing auxotrophic *S. cerevisiae* to grow to similar levels (Cagnac et al. 2004) but GSSG did not produce current in AtOPT6-expressing oocytes might be explained by reduction of GSSG to GSH before transport by *S. cerevisiae* under starvation conditions. *Saccharomyces cerevisiae* has been reported to reduce extracellular Fe(III) (Lesuisse and Labbe 1992), Cu(II) (Hassett and Kosman 1995) and azo dyes (Ramalho et al. 2005) via an inducible transmembrane redox system.

The results with tetra- and pentapeptides only indirectly suggest in planta AtOPT6 functions. The two known plant pentapeptides that were tested in AtOPT6-expressing oocytes, phytosulfokine and PC$_2$, incited either no current or only very small currents, making it unlikely that they are in planta substrates for AtOPT6. Although AtOPT6 and AtOPT4 transport tetra- and pentapeptides with substantial currents, and substrates in this group are transported with similar high affinity for both, it remains to be seen if any tetra- and pentapeptides that occur in plants are transported by AtOPT6 or AtOPT4. Tetra- and pentapeptides could perform either signaling or nutritional roles, e.g. in the distribution of mobilized protein storage reserves (Stacey et al. 2006).

The transport of longer peptides that are involved in plant development or nematode pathogenicity, AtCLE19p12, AtCLE5p12, HgCLEp12 and M116D10p13, suggests that AtOPT6 may play a role in peptide signaling. These natural substrates were transported at micromolar concentrations. Unlike the study on *C. albicans* OPT genes, where *C. albicans* strains with multiple OPT gene knock-outs were compared with the wild type in terms of growth on peptide substrates (Reuß and Morschhäuser 2006), we showed that a single OPT transporter can transport a large variety of longer peptides. However, the plant requirement for CLE transmembrane transport is not clear because CLV3 has been shown to bind to the ectodomain of the plasma membrane receptor-like kinase CLV1 (Ogawa et al. 2008, Ohyama et al. 2009), although M116D10 has been reported to act intracellularly (Huang et al. 2006). In seedling culture very low concentrations of 1 µM CLV3 (Kondo et al. 2006) and 10 µM AtCle19 inhibit root growth both by impairing cell division and by altering cell layer identity specification (Fiers et al. 2005). Transmembrane transport possibly could be required as part of a tight regulation system to remove CLEs rapidly from access to receptors.

A clear function for AtOPT6-mediated peptide transport would be in systemic peptide movement. While no evidence for systemic movement of CLE peptides currently exists, the *Lotus japonicus* HAR1 receptor-like kinase controls autoregulation of root development and nodulation from the shoot, and a small CLE-like peptide has been proposed as a mobile signal that travels in the xylem from root to shoot (Krusell et al. 2002, Nishimura et al. 2002). In this case, a peptide transporter related to AtOPT6 could be required for loading into the xylem. Another mobile signal may subsequently travel in the phloem from shoot to root where TML
regulates nodulation (Magori et al. 2009). Other 12 amino acid peptides that could be tested for transport by AtOPT6 in oocytes include the product of the soybean early nodulation gene ENOD40 (Röhrig et al. 2002).

Depending upon the amount of overlap in plant OPT substrate selectivity, altering the transport of GSH, tetrapeptides and pentapeptides and CLE-like peptides by AtOPT6 could have varied physiological effects. To date no clear phenotype has been identified in AtOPT6 knock-out lines, although they have not been fully characterized yet. In addition, we have shown that the selectivity of OPTs for peptides is difficult to predict. Large differences in substrate specificities of ScOPT1 and AtOPT6 show that sequence homology to other OPTs does not predict transport properties. Also, the length of peptide substrates only loosely predicted transportability by AtOPT6, resulting in an unwieldy number of possible substrates. A full characterization of OPT function in Arabidopsis awaits phenotyping of mutant lines in which multiple AtOPT genes are knocked out. In addition, structure/function studies would determine OPT substrate selectivity domains and perhaps identify selectivity filter sequences that allow prediction of substrate selectivity of functionally uncharacterized OPTs. Our characterization by two-electrode voltage clamp experiments of a number of peptide substrates, of different peptide lengths and sequences, and of markedly different patterns of transport for AtOPT6 and ScOPT1 lays ample ground for structure/function studies.

**Materials and Methods**

**Oocyte isolation and maintenance**

Oocytes were isolated and maintained as described in Osawa et al. (2006) with the following modifications: oocytes were defolliculated for 30–90 min and were injected with 23–46 ng of ScOPT1 or AtOPT6 cRNA on the same day or the day following isolation. Following injection, oocytes were incubated in an ND-96 Ringer solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 5 mM HEPES, 2.5 mM sodium pyruvate, pH 7.4) supplemented with antibiotics (10 µg ml⁻¹ streptomycin sulfate and 50 µg ml⁻¹ gentamycin sulfate).

**Two-electrode voltage clamp**

Oocytes were voltage clamped 4–6 d after cRNA injection in a bath solution containing 210 mM sorbitol, 6 mM MgCl₂, 1.8 mM CaCl₂, and 5 mM MES/Tris (pH 5.0, unless otherwise noted). Peptides were applied in the bath solution at a concentration of 500 µM, pH 5.0, unless otherwise noted. Uninjected control oocytes exhibited on average 0.37 ± 2.4 nA (n = 41) inward current when 500 µM GSH at pH 5 was applied in the bath (holding potential −80 mV). GSH applied to oocytes injected with AtOPT6 cRNA caused an inward current greater than that in uninjected oocytes (P < 0.5) in about 50% of measurable oocytes (sample = 102 oocytes).

To increase the likelihood that an oocyte was expressing AtOPT6, 500 µM GSH was applied as the first substrate, and those oocytes with −9 nA or more GSH inward current were used for other substrates after washing out GSH for 5 min and testing that pipetted bath solution did not induce current. Multiple additional substrates were applied to each oocyte, similarly washing between treatments. The average GSH current for all experiments was −22.6 ± 12.3 nA (n = 65). A second application of GSH to the same oocyte, immediately or following other substrates, produced currents that were not statistically different at the 0.5 level (data not shown). The response to GSH correlated significantly (P < 0.05) for some substrates that produced currents (e.g. PC2, 0.99; GGFL, 0.96) but was not correlated with others (e.g. KLGL, KLLLG, DRVYIHPF and AtCle19p12) and could not be used to normalize data.

**Sources for peptides and peptide-like substrates**

GSH, GSSG, GNOS and ‘EC were purchased from Sigma-Aldrich (St Louis, MO, USA). RVSPGGPDQHH (AtCLES), RVIPTPGNPLHN (AtCLE19), RLPSGPDPHHH (H. glycines CLE-like peptide) and GKKPPGPNPPGNN (M. incognita Mi16D10p13 pathogenesis protein) were custom-synthesized by Sigma-Genosys (The Woodlands, TX, USA) and were a kind gift of Dr. Melissa Mitchum (University of Missouri). The peptides GGFL, RPPGF, RPPGFSP, RPPGFSPFR, DRVYIHPF and DRVYIHPFHL were purchased from the American Peptide Co. (Sunnyvale, CA, USA). GGFM and KG were purchased from Bachem (King of Prussia, PA, USA). The peptides KLG, KLGL, KLLG, KLLLG, KLLLLG, GLLLK and PC₂ were synthesized by Dr. Fabio Gallazzi (University of Missouri Structural Biology Core). All custom-synthesized and purchased peptides were tested for purity by HPLC and mass spectrometry. Jasmonate—isolucine was a kind gift of Dr. Paul Staswick (University of Nebraska-Lincoln), and phytosulfinokine was a kind gift of Dr. Yoshikatsu Matsubayashi (Nagoya University).

**Statistics**

SAS statistical software (SAS Institute, Inc., Cary, NC, USA) was used to analyze data. PROC MEANS was used to conduct a paired t-test of the null hypothesis that the current caused by application of 500 µM GSH did not differ before and after such application of GSH at P ≤ 0.05 (above). PROC REG was used to conduct a regression analysis of the influence of pH on current incubated by 1 mM GSH in oocytes expressing AtOPT6 and uninjected oocytes (Fig. 2B). PROC GLM was used to conduct analysis of variance (ANOVA) with mean separation by Tukey’s Studentized range test at P ≤ 0.05 on currents caused by the application of peptides in oocytes expressing AtOPT6 and uninjected oocytes (Figs. 1, 3, 4A, 5, 6, 7 and above).
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References


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