AtPTR1 and AtPTR5 Transport Dipeptides in Planta[1][OA]

Nataliya Y. Komarova, Kathrin Thor[2], Adrian Gubler, Stefan Meier, Daniela Dietrich[3], Annett Weichert, Marianne Suter Grotemeyer, Mechthild Tegeder, and Doris Rentsch*

Molecular Plant Physiology, Institute of Plant Sciences, University of Bern, 3013 Bern, Switzerland (N.Y.K., K.T., A.G., S.M., D.D., A.W., M.S.G., D.R.); and School of Biological Sciences, Washington State University, Pullman, Washington 99164 (M.T.)

Transporters for di- and tripeptides belong to the large and poorly characterized PTR/NRT1 (peptide transporter/nitrate transporter 1) family. A new member of this gene family, *AtPTR5*, was isolated from Arabidopsis (*Arabidopsis thaliana*). Expression of *AtPTR5* was analyzed and compared with tissue specificity of the closely related *AtPTR1* to discern their roles in planta. Both transporters facilitate transport of dipeptides with high affinity and are localized at the plasma membrane. Mutants, double mutants, and overexpressing lines were exposed to several dipeptides, including toxic peptides, to analyze how the modified transporter expression affects pollen germination, growth of pollen tubes, root, and shoot. Analysis of *atptr5* mutants and *AtPTR5*-overexpressing lines showed that AtPTR5 facilitates peptide transport into germinating pollen and possibly into maturating pollen, ovules, and seeds. In contrast, AtPTR1 plays a role in uptake of peptides by roots indicated by reduced nitrogen (N) levels and reduced growth of *atptr1* mutants on medium with dipeptides as the sole N source. Furthermore, overexpression of *AtPTR5* resulted in enhanced shoot growth and increased N content. The function in peptide uptake was further confirmed with toxic peptides, which inhibited growth. The results show that closely related members of the PTR/NRT1 family have different functions in planta. This study also provides evidence that the use of organic N is not restricted to amino acids, but that dipeptides should be considered as a N source and transport form in plants.

Plants have evolved multiple transport systems for nitrogen (N) to facilitate uptake from soil and internal reallocation to support development, growth, and reproduction (Glass et al., 2002; Rentsch et al., 2007). Although organic N is generally the most prevalent form of N in the soil, it is considered to be a N source for soil microorganisms rather than plants (Kaye and Hart, 1997). However, the view that plants rely entirely on microorganisms for mineralization of organic N has been revised. Plant roots can take up amino acids (Wright, 1962) and the ecological significance of this ability has been recognized (Chapin et al., 1993; Näsholm et al., 1998; Schmidt and Stewart, 1999). Studies with Arabidopsis (*Arabidopsis thaliana*) mutants *atlht1* and *ataap1* provided molecular evidence that amino acid transporters facilitate uptake of amino acids into roots (Hirner et al., 2006; Lee et al., 2007; Svennerstam et al., 2007). In addition to amino acids, more complex organic N sources may also be important. There is evidence that plant roots access proteins as a N source without relying on soil microbes or symbiotic fungi (Paungfoo-Lonhienne et al., 2008). Likely mechanisms for protein acquisition include exudation of proteolytic enzymes at the root surface and possibly in the root apoplast and uptake of protein via endocytosis (Paungfoo-Lonhienne et al., 2008). Thus, peptides and proteins may be more important N sources for plants than previously considered. Plants were shown to be able to grow on small peptides, but it was not established whether hydrolysis occurred prior to uptake (Higgins and Payne, 1982).

Rather than being considered a N source for growth, small peptides are viewed in the context of germination and senescence, which require rapid proteolysis and retranslocation of N (Higgins and Payne, 1982). Protein degradation products from the endosperm are transported as di- and oligopeptides into the scutellum of germinating barley (*Hordeum vulgare*) grains to supply the embryo (Higgins and Payne, 1978). Rates of peptide transport into the scutellum were higher than transport rates of amino acids, which highlights the importance of peptides for supplying sinks (Higgins and Payne, 1978). Peptide transport also occurs in mature broad bean (*Vicia faba*) leaves (Jamai et al., 1994), which in turn demonstrates that peptide transport is not restricted to specific developmental stages. Rather, mobilization of peptides from proteolysis and transport of peptides throughout the plant appears to be an efficient strategy for rapid reallocation of N.

Data available on concentration of small peptides in soil and plants are limited, mainly hampered by the

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[2] Present address: Institute of Plant Sciences, ETH Zurich, Universitätstrasse 2, 8092 Zurich, Switzerland.

[3] Present address: Centre for Plant Integrative Biology, University of Nottingham, Sutton Bonington Campus, Loughborough LE12 5RD, UK.

* Corresponding author; e-mail doris.rentsch@ips.unibe.ch.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Doris Rentsch (doris.rentsch@ips.unibe.ch).

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lack of suitable detection systems. Breakdown of proteins and the rapid conversion of peptides into amino acids are problematic issues when preparing biological samples for determining the concentration of small peptides. Pools of small peptides have been reported, but the size and composition of these pools were determined only in few cases (Higgins and Payne, 1982). The least ambiguous evidence for a significant amount of peptides comes from studies with germinating barley grains, which demonstrated a pool of peptides of two to six amino acids with similar composition to seed storage proteins (Higgins and Payne, 1981, 1982).

Peptide transporters that have been identified in plants belong to three gene families, each recognizing peptides of defined length. Di- and tripeptides are transported by members of the PTR/NRT1 (peptide transporter/nitrate transporter 1) family, transport of peptides of four to five amino acids is mediated by members of the OPT (oligopeptide transporter) family, and transporters for larger peptides, so far only characterized in animals, have been identified among the ATP-binding cassette (ABC) transporters (Rentsch et al., 2007).

In prokaryotes, fungi, and animals, the PTR/NRT1 gene family has few members, while in plants this gene family is much larger. Arabidopsis has 53 genes in four subfamilies of PTR/NRT1 (Fig. 1; Tsay et al., 2007). For most PTR/NRT1 transporters in plants, substrate selectivity has not been determined and some PTR/NRT1 proteins mediate transport of substrates other than peptides (i.e. uptake or export of nitrate or carboxylates; Jeong et al., 2004; Segonzac et al., 2007; Tsay et al., 2007). Several members of subfamily II (see below) and one member of subgroup III (AtPTR3, At5g46050) are confirmed transporters for di- and tripeptides (Karim et al., 2005, 2007; Rentsch et al., 2007). Species with confirmed peptide transporters in subgroup II include Arabidopsis (AtPTR1, AtPTR2), fava bean (VfPTR1), *Hakea actities* (HaPTR4), and barley (HvPTR1; Frommer et al., 1994; Rentsch et al., 1995; West et al., 1998; Miranda et al., 2003; Dietrich et al., 2004). Subfamily II also contains several noncharacterized genes from Arabidopsis and other species (Fig. 1).

AtPTR1 and AtPTR2 recognize various di- and tripeptides with high affinity (Rentsch et al., 1995; Chiang et al., 2004; Dietrich et al., 2004). Chiang et al. (2004) also established that AtPTR2 transports peptides and protons simultaneously by a random binding mechanism. Evidence for a physiological role of these di-/tripeptide transporters in plants is limited to antisense repression of *AtPTR2*, which showed that peptide transport is important for flowering and seed growth (Song et al., 1997).

Linking functional characterization of peptide transporters in heterologous expression systems with physiological evidence for peptide transport, we isolated

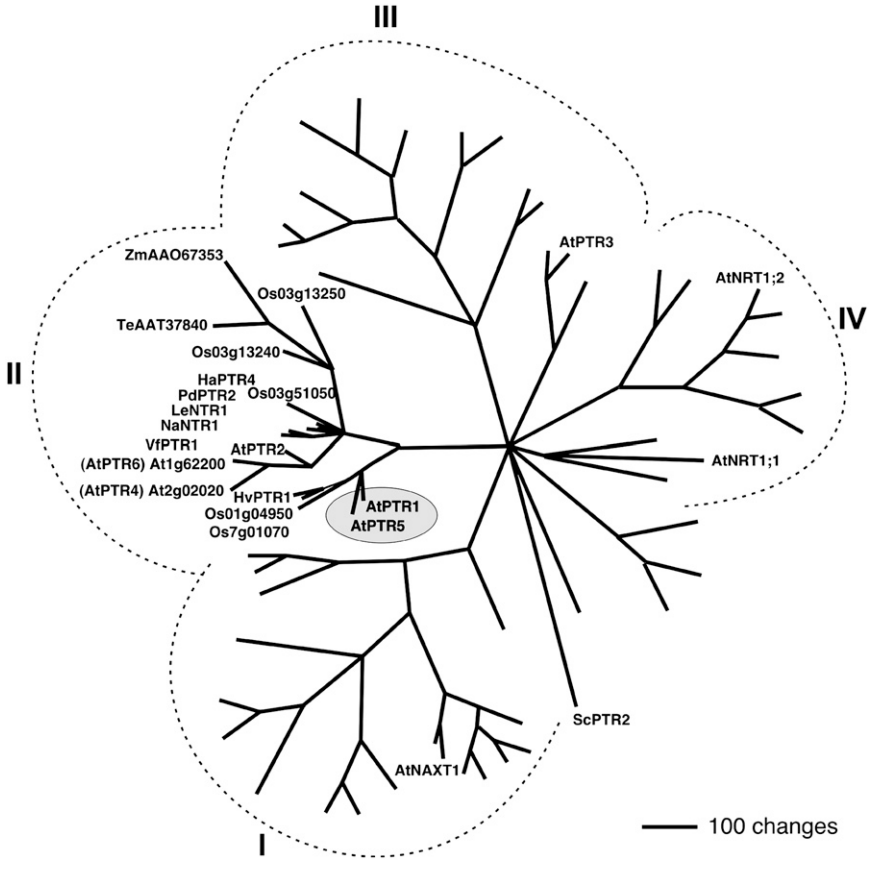

Figure 1. Phylogenetic relationship between AtPTR1, AtPTR5, and related proteins. The analysis was performed using the aligned protein sequences of the Arabidopsis PTR/NRT1 gene family, including proteins from other plant species for proteins of subfamily II (PTR1-like proteins; Rentsch et al., 2007; Tsay et al., 2007; VfPTR1, Miranda et al., 2003; NaNTR1, Schulze et al., 1999; HaPTR4, C. Paungfoo-Lonhienne, D. Rentsch, and S. Schmidt, unpublished data; PdPTR2, Campalans et al., 2001; LeNTR1, AF016713; HvPTR1, West et al., 1998). Maximum parsimony analysis was performed using PAUP 4.0b10 with all characters unweighted and gaps scored as missing characters (Swofford, 2003). The complete alignment was based on 1,409 amino acids, 633 characters were parsimony informative. The yeast peptide transporter ScPTR2 was used as the outgroup.

and characterized the Arabidopsis di-/tripeptide transporter AtPTR5. We demonstrate that AtPTR5 mediates uptake of peptides during pollen germination. Further, expression and localization studies suggest a function of AtPTR5 in N transport during ovule and early seed development. Using *atptr* mutants and *AtPTR5*-overexpressing lines, we also provide evidence that peptide transporters facilitate uptake of N from the rhizosphere.

RESULTS

AtPTR5 Transports Dipeptides When Expressed in Yeast and *Xenopus* Oocytes

AtPTR5 (At5g01180) belongs to subgroup II of the PTR/NRT1 family and has highest homology to AtPTR1 (74% identity on amino acid level), to barley HvPTR1 (64.7% identity), and two noncharacterized open reading frames (ORFs) from rice (*Oryza sativa*; Os01g04950, 67% identity and Os7g01070, 62% identity; Fig. 1; Tsay et al., 2007). An *AtPTR5*-ORF was isolated by reverse transcription (RT)-PCR using total RNA of Arabidopsis flowers. Expression of AtPTR5 in the peptide transport-deficient and His auxotroph yeast (*Saccharomyces cerevisiae*) strain LR2 showed that, similar to other members of subfamily II, AtPTR5 mediates growth when using selective concentrations of His-Ala (1 mM) as sole source of His (Fig. 2; Frommer et al., 1994; Rentsch et al., 1995; West et al., 1998; Miranda et al., 2003; Dietrich et al., 2004). Interestingly, the Arabidopsis di- and tripeptide transporters AtPTR1 and AtPTR2 also mediated growth on selective concentrations of His (6 mM), although affinity for His and transport rates seem to be rather low (Frommer et al., 1994; Chiang et al., 2004; Dietrich et al., 2004). In contrast, AtPTR5 did not mediate growth on 6 mM His (Fig. 2). As control, vector-transformed cells and LR2 expressing the amino acid permease AtAAP2 that mediates growth on His, but not on dipeptides, is shown (Kwart et al., 1993; Rentsch et al., 1995; Dietrich et al., 2004).

To compare the kinetic properties of AtPTR5 with other peptide transporters, dipeptide-induced currents of neutral, basic, and acidic amino acid-containing dipeptides were analyzed in *Xenopus laevis* oocytes. At a membrane potential of −140 mV and $[H^+]_{out}$ of 3.2 μM (pH 5.5), the addition of 1 mM Ala-Ala, Ala-Lys, and Ala-Asp to the bathing medium induced inward currents with a range of 70 to 740 nA in oocytes expressing AtPTR5. No dipeptide-induced currents were observed in control oocytes. Peptide-induced inward currents were voltage dependent, increasing with hyperpolarizing membrane potential within the range of −140 mV to +20 mV (data not shown). At −140 mV and $[H^+]_{out}$ of 3.2 μM, the apparent affinity of AtPTR5 for Ala-Asp ($K_{0.5}^{Ala\text{-}Asp}$ 131 ± 1 μM) and Ala-Lys ($K_{0.5}^{Ala\text{-}Lys}$ 163 ± 7 μM) was similar to the affinity determined for other di-/tripeptide transporters (Chiang et al., 2004; Dietrich et al., 2004).

AtPTR5 Is Localized at the Plasma Membrane

Immunodetection of HvPTR1 on isolated plasma membranes or expression of AtPTR1-GFP and GFP-AtPTR1 fusion proteins have been used to demonstrate the localization at the plasma membrane (Waterworth et al., 2000; Dietrich et al., 2004). Functional complementation of the yeast peptide transport mutant LR2 by AtPTR5 and functional expression of AtPTR5 in *X. laevis* oocytes indicated that at least part of the AtPTR5 protein is localized at the plasma membrane. To corroborate evidence for localization, N- and C-terminal fusion proteins of AtPTR5 and GFP were transiently expressed in tobacco (*Nicotiana tabacum*) protoplasts. Fluorescence images showed that AtPTR5-GFP and GFP-AtPTR5 fusion proteins localize at the plasma membrane (Fig. 3, A and C, respectively). Free GFP

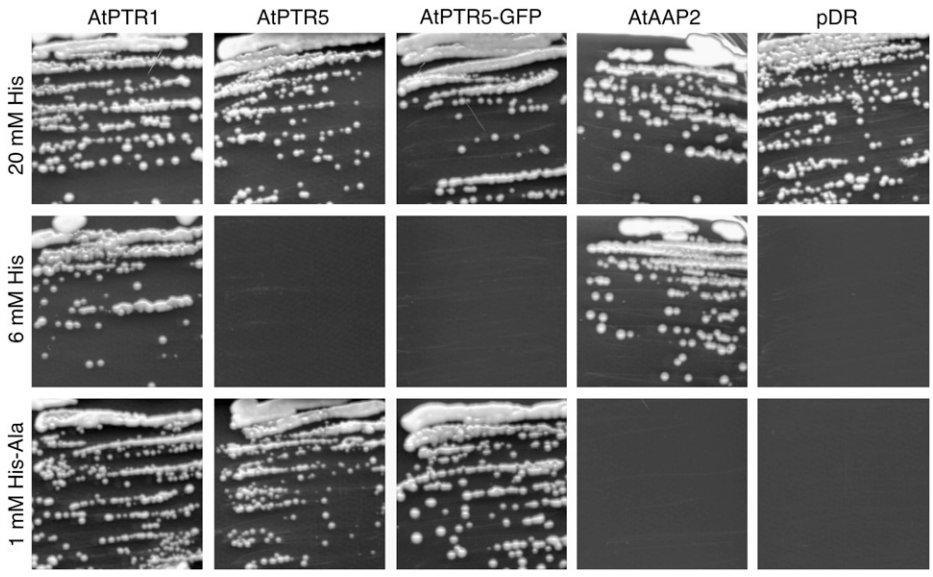

Figure 2. Complementation of a yeast strain (LR2) deficient in the uptake of di- and tripeptides. Shown is growth of LR2 cells expressing the peptide transporters AtPTR1 (Dietrich et al., 2004), AtPTR5, and AtPTR5-GFP, the amino acid transporter AtAAP2 (Kwart et al., 1993), and the strain transformed with the vector pDR195 on SC medium containing selective concentrations of His (i.e. 6 mM His or 1 mM His-Ala) or nonselective His concentrations (20 mM).

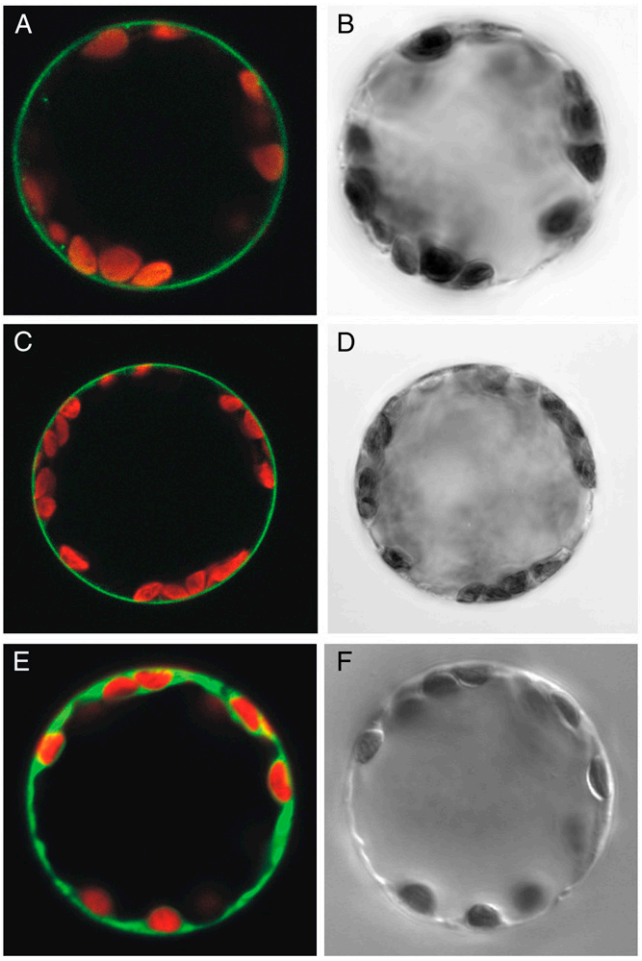

Figure 3. Localization of AtPTR5/GFP fusion proteins at the plasma membrane of tobacco protoplasts. Confocal laser-scanning microscope images (A, C, E) and corresponding bright-field images (B, D, F) of tobacco protoplasts transiently expressing fusion proteins of GFP with AtPTR5 or GFP. A and B, AtPTR5-GFP. C and D, GFP-AtPTR5. E and F, GFP. Merged images show GFP fluorescence (green) and chlorophyll fluorescence (red). Diameter of protoplasts is approximately 40 μm.

was localized in the cytosol (Fig. 3E). Fusion to GFP did not disturb peptide transport function because the AtPTR5-GFP fusion protein was functional when expressed in yeast (Fig. 2).

AtPTR5 Is Expressed in Pollen, Ovules, and during Early Seed Development

Semiquantitative RT-PCR using RNA from soil-grown plants showed weak *AtPTR5* expression in seedlings, flowers, and young siliques (Fig. 4A). This expression pattern was supported by analyses of 37 independent transgenic Arabidopsis lines, expressing the *Escherichia coli* GUS (*uidA*) gene under control of a 2,582-bp fragment of the *AtPTR5* promoter. All lines displayed highest GUS activity in mature and germinating pollen, ovules, and developing seeds (Fig. 4, D–H). This expression pattern was corroborated by publicly available microarray data (e.g. Genevestigator; Zimmermann et al., 2004). GUS activity decreased after the early torpedo stage of embryo development and was absent in mature and germinating seeds (Fig. 4H; data not shown). One-half of the lines had weak GUS activity in leaves of seedlings, and mature plants of all lines displayed weak GUS activity in leaves and older sections of roots (Fig. 4, B and C).

AtPTR1 and AtPTR5 Mediate Transport of Peptides in Planta

To assess the role of AtPTR5 and its closest homolog AtPTR1 for uptake of peptides from the growth medium and translocation in plants, we analyzed *atptr1* and *atptr5* mutants and transgenic Arabidopsis lines overexpressing *AtPTR5* under the control of the constitutive 35S promoter.

Atptr1-1 carries the T-DNA in the last exon, whereas *atptr1-2* has an insertion 220 bp upstream of the start codon, lying in an intron of the 5′-untranslated region (UTR; Fig. 5A). Both *atptr5* mutant lines carry an insertion in the last exon. Using total RNA from leaves (*AtPTR1*) or flowers (*AtPTR5*) and primers downstream of the insertion or enclosing the T-DNA (Fig. 5A), RT-PCR analysis verified that mRNA of *AtPTR1* and *AtPTR5* was absent in the *atptr1-1* and both *atptr5* mutant lines, as well as in the corresponding double mutants. In the *atptr1-2* single and *atptr1-2 atptr5-1* double mutant, mRNA levels of *AtPTR1* were only partially reduced (Fig. 5B). With primers upstream of the T-DNA insertion, *AtPTR5* and *AtPTR1* transcripts could be amplified in both *atptr5* lines and in the *atptr1-1* line (data not shown). As the partial mRNA could result in a truncated, but possibly still functional protein, a cDNA was constructed that reflects the genomic situation in the *atptr5-1* mutant and consisted of the truncated *AtPTR5*-ORF and part of the T-DNA. The cDNA was expressed in the yeast strain LR2. This truncated AtPTR5 was not able to restore growth of the yeast mutant, indicating that in the *atptr5-1* line AtPTR5-mediated peptide transport does not occur (data not shown).

In addition to the analysis of knockout mutants, 45 independent transgenic Arabidopsis lines overexpressing *AtPTR5* under control of the 35S promoter were analyzed for *AtPTR5* transcript levels. Two lines (T3 plants) with elevated *AtPTR5* mRNA in leaves were selected for further investigation (Fig. 5C). When cultured in soil or on AM medium, no growth phenotype (e.g. shoot growth, root length, flowering time) could be detected between wild type, single and double mutants, or overexpression lines. Furthermore, in all experiments, the corresponding single and double mutants behaved identically. Although the *atptr1-2* line showed only a partial reduction in *AtPTR1* transcript level in leaves, no difference to *atptr1-1* was found.

GUS analyses and publicly available microarray data (e.g. Genevestigator; Zimmermann et al., 2004) showed high expression of *AtPTR5* in pollen. To assess peptide transport activity during pollen germination,

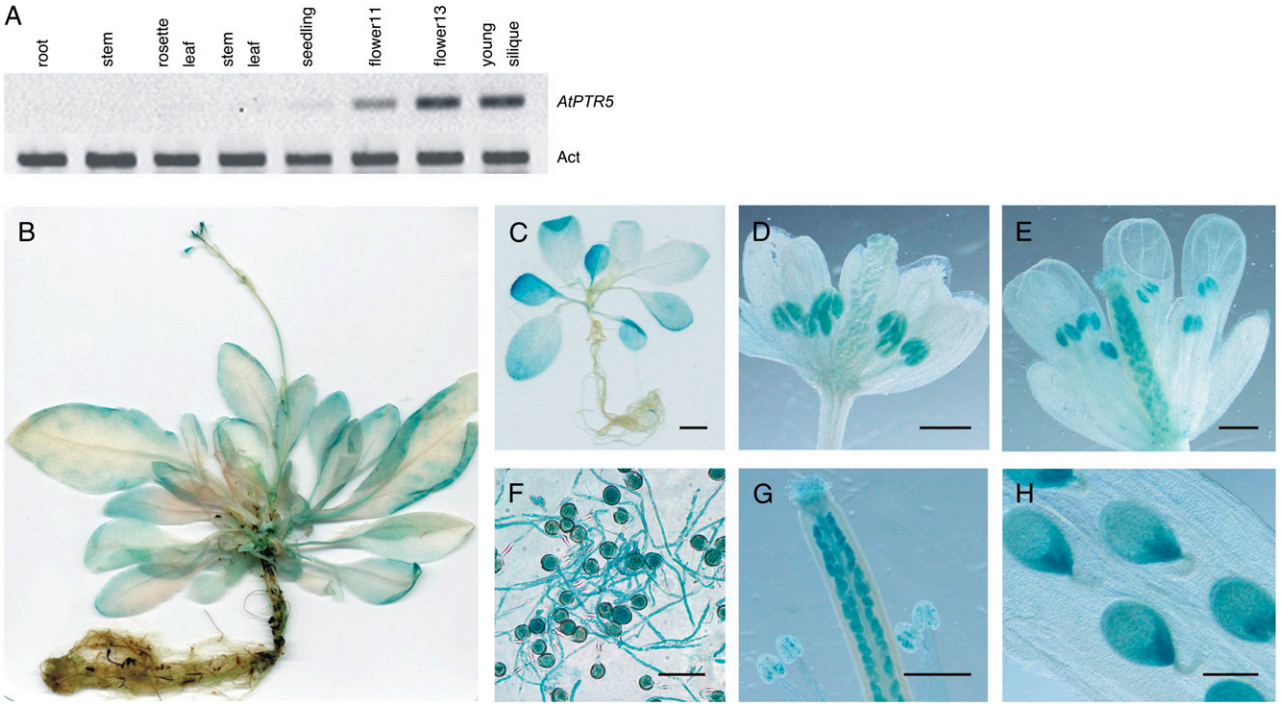

Figure 4. Expression analysis of *AtPTR5* in Arabidopsis. A, Expression was analyzed by using semiquantitative RT-PCR with the actin mRNA (Act, At3g18780) as a reference. RNA from source leaves, stems, roots, flowers, and siliques of soil-grown plants was analyzed. Similar results were obtained using RNAs from three independent experiments as template. B to H, Expression of the *uidA* gene under control of the *AtPTR5* promoter. *AtPTR5*-GUS expression in 6-week-old plants (B), 2-week-old seedlings (C), flowers before pollination (stage 12; D), and after pollination (stage 14; E), in vitro-germinated pollen (F), and developing seeds (G and H). No staining could be detected in mature seeds and during seed germination and seedling establishment. Bars = 2,000 μm in C, 25 μm in F, 200 μm in H, and 500 μm in D, E, and G.

pollen was germinated for 6 and 14 h in the presence of the toxic dipeptide alanyl-ethionine (Ala-Eth) and the effect on germination rate and pollen tube growth was determined. Germination was defined as protrusion of pollen tubes from the pollen grain, whereas elongation of the pollen tube defined growth. On germination medium (GM; Footitt et al., 2007), no differences were observed in germination rate and growth of pollen tubes of wild type and mutants (*atptr1-1*, *atptr1-2*, *atptr5-1*, *atptr5-2*, *atptr1-2 atptr5-1*, and 35S:*AtPTR5*a; Fig. 6, all lines mentioned in the text were tested, but only one of the lines is shown). In the presence of 100 μM Ala-Eth, germination was strongly reduced in the 35S:*AtPTR5*a line, whereas no difference was observed between wild-type pollen and pollen of the different knockout lines (Fig. 6A). On Ala-Eth-containing medium, growth of the pollen tube was least affected in both *atptr5* knockout lines. Reduction of pollen tube elongation was in the order 35S:*AtPTR5*a > (*atptr1-1*, *atptr1-2*, wild type) > (*atptr5-1*, *atptr5-2*, *atptr1-2 atptr5-1*), indicating that AtPTR5 mediates uptake of peptides during pollen germination and tube growth (Fig. 6B).

Although *AtPTR5* expression was detected in the ovule and early stages of seed development, this did not affect mature seeds of *atptr5*, 35S:*AtPTR5*a, and wild-type plants as they had a similar N content (data not shown).

In contrast to *AtPTR5*, *AtPTR1* expression was also detected in roots (Dietrich et al., 2004). In agreement with these findings, in the presence of the toxic dipeptide Ala-Eth, root growth of both *atptr1* lines and the *atptr1 atptr5* double mutants was less affected than wild type or the *atptr5* single mutants (Fig. 7, A–C). On AM medium, no differences in root growth could be observed among the lines (Fig. 7D). To investigate whether AtPTR1 and/or AtPTR5 is required for uptake of small peptides from the medium and whether these peptides can be used as the sole N source, wild-type, single and double mutants (*atptr1-1*, *atptr1-2*, *atptr5-1*, *atptr5-2*, *atptr1-1 atptr5-1*, *atptr1-2 atptr5-1*), as well as both 35S:*AtPTR5* lines were germinated on minimal medium (−N) containing 20 mM of a dipeptide as sole source of N (Fig. 8). After 2 weeks on AM control medium, no differences in shoot growth were observed between wild-type and mutant lines. On medium with any of the dipeptides as sole N source, all seedlings produced more anthocyanins and shoot growth was reduced, while thickness of shoots seemingly increased. On Ala-Ala, Ala-Asn, and Pro-Ala no difference in growth could be observed between wild type and both *atptr5* mutants, whereas *atptr1* single and *atptr1 atptr5* double mutants were more strongly reduced in shoot growth. No difference between the *atptr1* mutants, wild type, and the *atptr5* mutants was

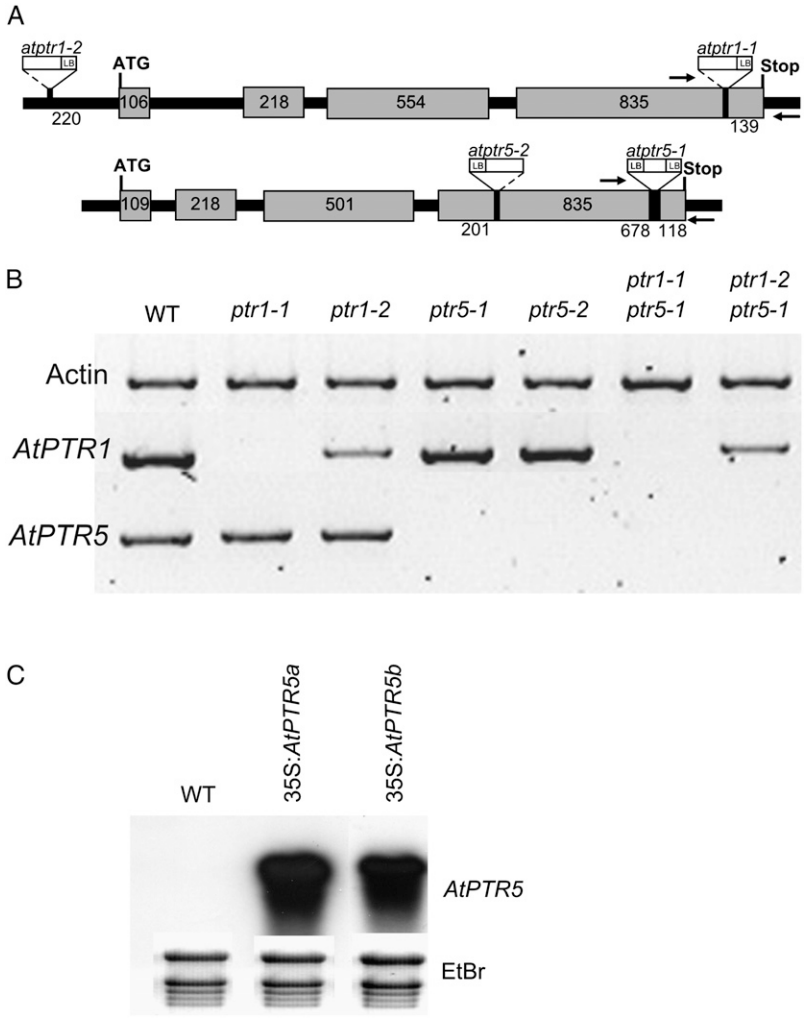

Figure 5. Characterization of homozygous *atptr1*, *atptr5*, and *atptr1 atptr5* mutants as well as overexpression lines (35S:*AtPTR5*). A, Position of T-DNA or transposon insertions in the *AtPTR1* or *AtPTR5* gene. Gray boxes represent exons. Numbers indicate length in base pairs for each exon or distances between exon borders and insertions. Arrows show position of primers used for RT-PCR in B. ATG, Start codon; Stop, stop codon; LB, left border of the insertion. B, Semiquantitative RT-PCR analysis of expression of *AtPTR1*, *AtPTR5*, and actin in wild type, the single mutants *atptr1-1*, *atptr1-2*, *atptr5-1*, and *atptr5-2*, and the double mutants *atptr1-1 atptr5-1* and *atptr1-2 atptr5-1* using RNA from flowers (*AtPTR5*) and leaves (*AtPTR1*). C, Northern-blot analysis of RNA from mature leaves of wild-type plants (WT) and 35S:*AtPTR5*-overexpressing lines (35S:*AtPTR5*a and 35S:*AtPTR5*b). As a loading control, staining with ethidium bromide (EtBr) is shown.

observed on Ala-Phe or Lys-Asp (Fig. 8). In contrast, growth of both overexpressing lines (35S:*AtPTR5*) was improved on Ala-Ala, Pro-Ala, and Ala-Asn and was strongly reduced on Ala-Phe and Lys-Asp (Fig. 8). As in soil, peptides are expected to be present as a mixture rather than as a single dipeptide, seedlings were also cultivated on a mixture of dipeptides (2.5 mM) in the presence or absence of low concentrations (0.1 mM) of ammonium nitrate (Fig. 9). This mixture contained five different dipeptides (0.5 mM each), consisting of amino acids that were described to be nontoxic at low concentrations (Voll et al., 2004; Lee et al., 2007). All lines grew equally well on control AM medium, growth was reduced on low ammonium nitrate, and poor on medium lacking N. In contrast, on medium containing dipeptides (with and without ammonium nitrate), overexpressing lines produced more leaves and were less chlorotic than wild type or knockout mutants, and wild type and *atptr5* mutants performed slightly better than the *atptr1* and *atptr1 atptr5* mutants. These visual phenotypic changes were confirmed by analysis of biomass. Compared to wild type, shoot dry weight of overexpressing lines was significantly increased up to 3-fold on Ala-Ala, Pro-Ala, and the peptide mixture, or decreased up to nearly 4-fold on Ala-Phe (Table I). In contrast, shoot dry weight was reduced by approximately 50% in *atptr1* and *atptr1 atptr5* seedlings on Ala-Ala and Pro-Ala. Shoot biomass of *atptr5* lines, on the other hand, was similar to wild type. Analysis of N content of shoots (% of dry weight) corroborates the evidence that changes in seedling growth and shoot dry weight are due to differences in N uptake (Fig. 10). N content of *atptr1* and *atptr1 atptr5* seedlings cultivated on dipeptides was significantly reduced, whereas the N content of *atptr5* lines was similar to wild type. Analysis of 35S:*AtPTR5* lines revealed an increase in shoot N content of up to 3-fold when grown on Pro-Ala as sole N source (Fig. 10). Shoot N content was also elevated when seedlings were cultivated on dipeptides in the presence of low concentrations of ammonium nitrate.

We examined whether amino acids resulting from extracellular cleavage of the dipeptides might lead to the differences observed. HPLC analyses of medium containing Ala-Ala or Ala-Pro showed that no amino acids were generated after 2 weeks in the absence or presence of plants (data not shown). Furthermore, on medium with an equimolar mixture of the amino acids Ala and Pro, no differences in growth, biomass, and

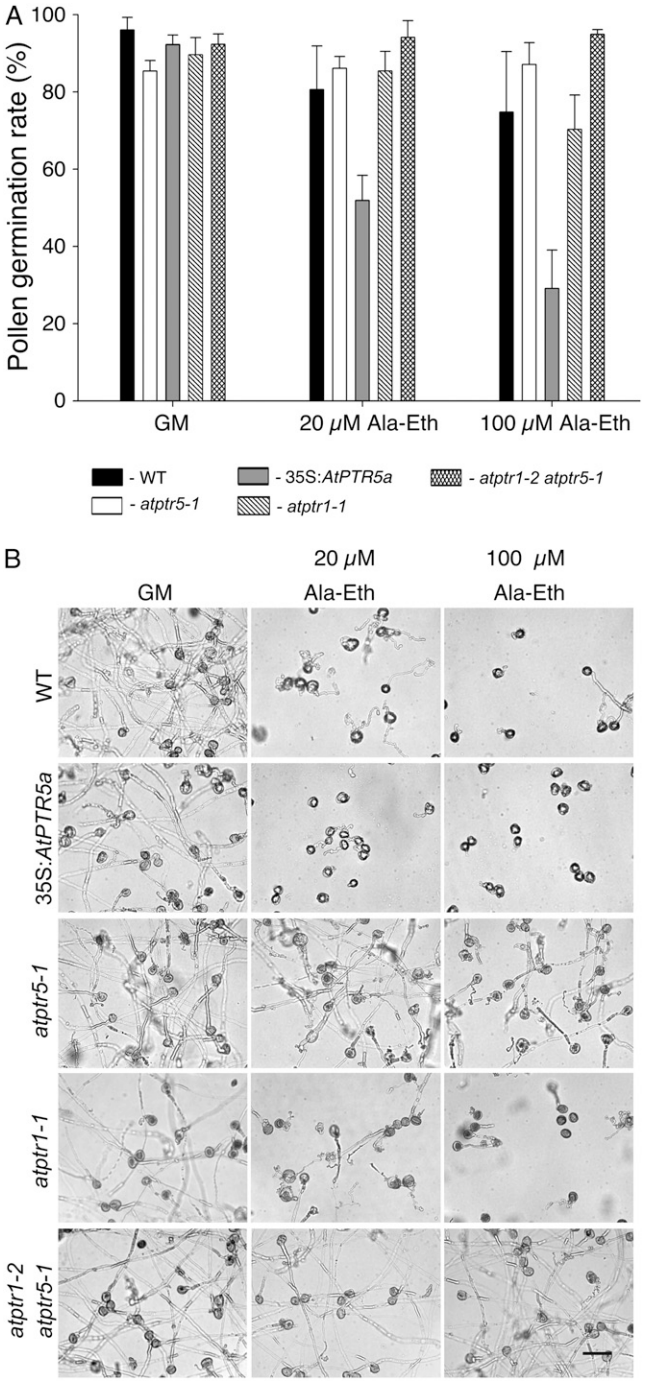

Figure 6. In vitro pollen germination of wild type, *atptr1-1*, *atptr5-1*, *atptr1-2 atptr5-1*, and 35S:*AtPTR5*a. A, Pollen germination rate of wild type, *atptr1-1*, *atptr5-1*, *atptr1-2 atptr5-1*, and 35S:*AtPTR5*a. Pollen was germinated for 14 h on GM (Footitt et al., 2007) in the absence or presence of 20 or 100 μM of the toxic dipeptide Ala-Eth. Germination was defined as emergence of the pollen tubes. B, Germination and growth of pollen tubes of wild type, *atptr1-1*, *atptr5-1*, *atptr1-2 atptr5-1*, and 35S:*AtPTR5*a; bar = 25 μm. Germination and pollen tube growth of *atptr1-2* and *atptr5-2* mutants was comparable to results shown for *atptr1-1* and *atptr5-1*, respectively (data not shown).

shoot N levels were observed between mutants, overexpressing lines, and wild type, while differences were seen for Pro-Ala (Figs. 8 and 10; Table I). This demonstrates that the observed growth differences on Pro-Ala were not due to extracellular cleavage of the dipeptide and subsequent uptake of amino acids, but originate from the uptake of the dipeptides; together, the data indicate that peptides are efficiently taken up and metabolized as a source of N.

DISCUSSION

AtPTR5 Mediates High-Affinity Transport of Dipeptides

AtPTR5 had similar apparent affinities for Ala-Lys ($K_{0.5}^{\text{Ala-Lys}}$ 163 μM) and Ala-Asp ($K_{0.5}^{\text{Ala-Asp}}$ 131 μM) as its closest homolog AtPTR1 ($K_{0.5}^{\text{Ala-Lys}}$ 112 μM, $K_{0.5}^{\text{Ala-Asp}}$ 416 μM; Dietrich et al., 2004), but a much higher affinity for Ala-Asp when compared to AtPTR2 ($K_{0.5}^{\text{Ala-Asp}}$ 1832 μM; Chiang et al., 2004). While the latter differences in $K_{0.5}$ values for Ala-Asp might be due to the higher membrane potential used for the electrophysiological measurements with AtPTR2, they most probably reflect true differences because transport of dipeptides by AtPTR2 seems to be largely independent of the membrane potential (Chiang et al., 2004). Nevertheless, $K_{0.5}$ values of AtPTR2 for other dipeptides composed of neutral and basic amino acids were similar to those determined for AtPTR5 and AtPTR1, indicating that affinities for peptides are generally conserved among the PTR transporters (Chiang et al., 2004; Dietrich et al., 2004).

AtPTR5 Is Localized at the Plasma Membrane and Facilitates Transport of Peptides into Reproductive Organs

AtPTR5 expression was detected in developing and germinating pollen grains as well as in ovules and during early stages of seed development (Fig. 4). Further, GFP fusion proteins showed that AtPTR5 is localized at the plasma membrane (Fig. 3). Together, these results indicate a role of AtPTR5 in uptake of di- and tripeptides into cells of reproductive organs. While AtPTR1 is also targeted to the plasma membrane, proteome analyses suggest that AtPTR2 is localized at the tonoplast, which points to a role in intracellular rather than intercellular transport (Carter et al., 2004; Dietrich et al., 2004; Shimaoka et al., 2004; Dunkley et al., 2006). The more distantly related AtPTR3 in subgroup III mediates transport in a peptide transport-deficient yeast strain, but affinities or intracellular localization have not been studied (Karim et al., 2005, 2007). Additionally, AtPTR3 seems to be involved in defense reactions during abiotic and biotic stresses rather than in transport of organic N during plant growth (Karim et al., 2007).

Partitioning of N to reproductive structures is a key determinant for development of viable pollen and seeds and thus for yield and successful reproduction

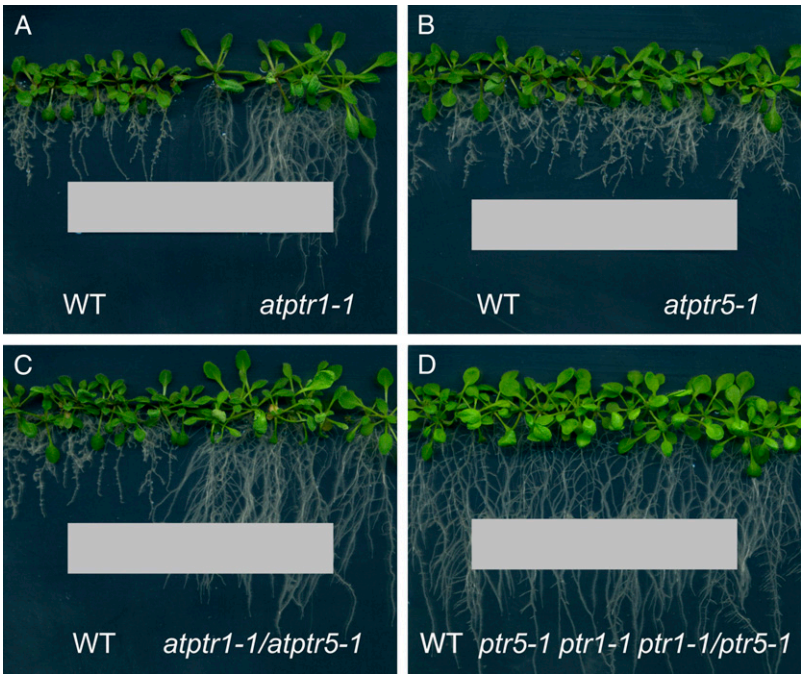

Figure 7. Root growth of Arabidopsis wild-type (WT), *atptr1-1*, *atptr5-1*, and *atptr1-1 atptr5-1* seedlings in the presence of the toxic dipeptide Ala-Eth. Arabidopsis plants were grown for 7 d on AM medium and subsequently for 10 d on AM medium containing a filter strip soaked with 1.1 μmol Ala-Eth (A–C) or with water (D). Data shown are representative for the independent single and double mutants.

(Pate, 1980; Gifford et al., 1984). During development and germination, pollen remains symplastically isolated from the surrounding tissue and depends entirely on nutrients supplied via the phloem from source tissues and their uptake from the apoplast. Studies of the Arabidopsis pollen transcriptome and localization analyzes identified several amino acid transporters, mainly of the *LHT* family, Pro transporter *AtProT1*, several members of the OPT family, but also *AtPTR5* among the genes expressed specifically or preferentially in pollen, supporting the importance of N supply for pollen production (Fig. 4; Honys and Twell, 2004; Lee and Tegeder, 2004; Pina et al., 2005; Bock et al., 2006). *AtPTR5* is expressed both in early and late stages with highest expression at the trinuclear stage of pollen development (Honys and Twell, 2004; Bock et al., 2006). The pollen germination assay showed that AtPTR5-mediated peptide uptake activity is present during early stages of germination (Fig. 6A). In addition, growth of the pollen tubes was negatively affected by Ala-Eth, indicating that AtPTR5-mediated peptide transport also occurs during pollen tube growth (Fig. 6B). However, the latter effect could also be explained by uptake of Ala-Eth during germination that subsequently affects growth of pollen tubes. Therefore, protein localization studies are needed to further determine whether AtPTR5 contributes to N transport during pollen germination and pollen tube growth.

AtPTR5-GUS activity was also detected in ovules and during early stages of seed development, suggesting a role of AtPTR5 in delivery of organic N for establishing the next generation (Fig. 4). Seed loading with N occurs mainly by organic N forms that are delivered via the phloem (Pate et al., 1977; Pate, 1980). Several amino acid transport systems are involved during this phase. *Ataap8* mutants showed that AtAAP8 is important for early seed development, while expression of *AtAAP1* and *AtAAP2* points to a complementary role during seed development (Hirner et al., 1998; Schmidt et al., 2007). Further, the oligopeptide transporter *atopt3* mutant showed arrested seed development; however, as recently demonstrated, AtOPT3 seems to be involved in iron transport rather than transfer of organic N (Stacey et al., 2002, 2008). With respect to dipeptide transport, arrested seed development has been described for *atptr2* antisense lines (Song et al., 1997). However, this effect could not be observed in *atptr2* T-DNA insertion lines, indicating that down-regulation of other *PTR*s in the antisense lines might have contributed to this phenotype (D. Dietrich and D. Rentsch, unpublished data). Similar to the amino acid transporters *AtAAP1* and *AtAAP2*, the dipeptide transporters *AtPTR1* and *AtPTR5* were expressed in the silique vasculature and developing seeds, with their expression preceding the protein-filling phase, suggesting a role in di- and tripeptide supply to and uptake into the seeds, respectively (Fig. 4; Hirner et al., 1998; Dietrich et al., 2004). Nonetheless, mature seeds of *atptr5* and wild-type plants did not differ in their total N content, indicating that AtPTR5 mediated di- and tripeptide transport does not represent a limiting step for final N content in seeds and/or that missing AtPTR5 activity is being compensated by other peptide or amino acid transporters.

Peptide Transporters Mediate Uptake of Peptides into Roots

Most soils contain a high content of organic N compounds that are, however, hardly accessible for

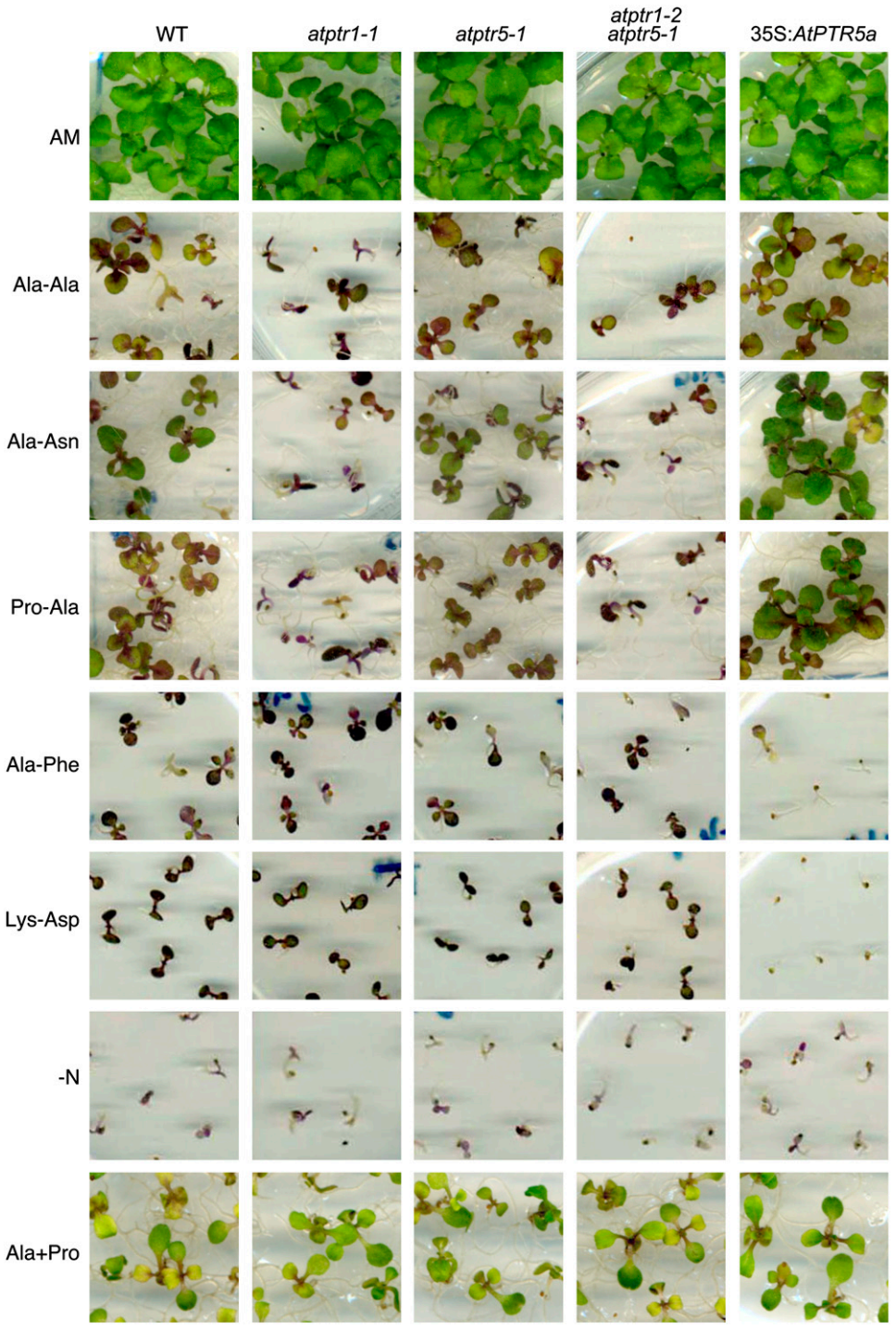

Figure 8. Growth of Arabidopsis wild-type (WT), *atptr1-1*, *atptr5-1*, *atptr1-2 atptr5-1*, and 35S:*AtPTR5*a seedlings on medium containing peptides as sole N source. Seeds were germinated and plants cultivated for 2 weeks either on AM medium or on one-half-strength Murashige and Skoog medium lacking NH_4NO_3, and KNO_3 replaced by KCl (−N) in the presence or absence of 20 mM of a dipeptide as a sole N source. Alternatively, seedlings were cultivated on −N medium supplemented with 10 mM Pro and 10 mM Ala (+Ala+Pro). Data shown are representative for the independent mutants and overexpressing lines, respectively.

degradation because they are protected by minerals and humus (Leinweber and Schulten, 1998). Proteolysis is generally considered as the rate-limiting step in protein degradation and released organic N is used by microorganisms and plants as N sources (Cunningham and Wetzel, 1989; Asmar et al., 1994). Paungfoo-Lonhienne et al. (2008) showed that plant roots can use larger proteins as N sources and that the proteins are hydrolyzed at the root surface and potentially in the root apoplast. While in these studies the sizes of the peptide fragments generated were not determined, import of peptides into root cells could potentially be mediated by transporters of the PTR, OPT, and/or ABC transporter families.

Here, we present evidence for uptake of dipeptides into roots by members of the PTR family. We demonstrate that *atptr1* mutants exhibit reduced growth and lower N content when cultivated on Ala-Ala, Pro-Ala,

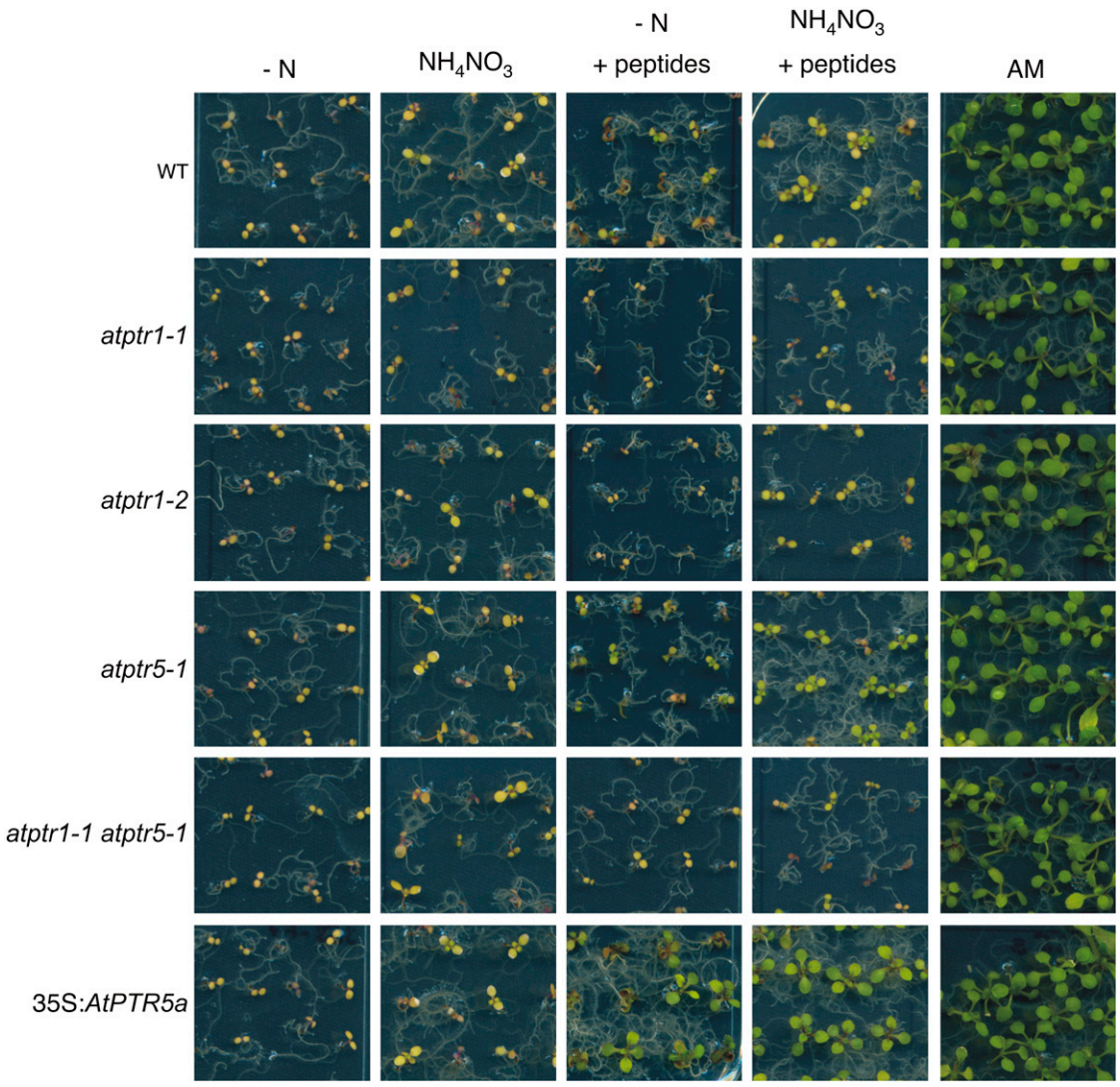

Figure 9. Growth of wild-type (WT), *atptr1-1*, *atptr1-2*, *atptr5-1*, *atptr1-1 atptr5-1*, and 35S:*AtPTR5*a seedlings on medium containing a mixture of peptides. Seeds were germinated and cultivated for 2 weeks either on AM medium or on one-half-strength Murashige and Skoog medium lacking NH_4NO_3 and KNO_3 replaced by KCl (−N) or on −N medium containing 0.1 mM NH_4NO_3 (NH_4NO_3) in the presence or absence of 2.5 mM of a peptide mixture (0.5 mM each of Ala-Ala, Pro-Ala, Ala-Asp, Arg-Glu, and Ala-Gly). Data shown are representative for the independent mutants and overexpressing lines, respectively.

or a mixture of dipeptides, supporting a function of AtPTR1 in peptide acquisition (Figs. 8–10; Table I). Conversely, when overexpressing a peptide transporter (35S:*AtPTR5*), plant growth was enhanced and N content increased (Fig. 8–10; Table I). Because *AtPTR5* is not expressed in roots, as expected, *atptr5* mutants behaved like wild-type plants. Under the conditions tested, *AtPTR5* did not seem to be upregulated in the absence of *AtPTR1* because the double mutants behaved like the single *atptr1* mutants. Whereas certain dipeptides, including Ala-Ala, Ala-Asn, and Pro-Ala promoted better growth of *AtPTR5*-overexpressing lines, Ala-Phe and Lys-Asp inhibited growth, indicating that their increased uptake caused a toxic effect either of the peptides or more likely by the amino acids generated (Fig. 8; Table I). Imbalance of the amino acid pools disturbs homeostasis, which can be lethal for the plant, a fact that was used to identify mutants in amino acid metabolism and transport (Voll et al., 2004; Lee et al., 2007). The predicted peptide transport activity 35S:*AtPTR5* > wild type > *atptr1* mutants correlated with the observed effect, indicating the dependence of the phenotypes on peptide transporter function. Because AtPTR1 did not mediate transport of the amino acids Ala, Lys, Asp, or Asn, and because His was only transported with low affinity (Dietrich et al., 2004), uptake of amino acids generated by extracellular cleavage and subsequent transport by a peptide transporter seemed unlikely, even more so because no amino acids could be detected in the growth medium. This notion was corroborated by the finding that growth of *atptr1*

Table I. *Shoot dry weight (DW) of 2-week-old Arabidopsis seedlings grown on medium with different N sources*

Medium and experimental setup are as described in the Figure 8 and 9 legends. Data were obtained from three independent experiments. Values are the means ± SE per plant. Different letters indicate statistically significant differences compared to wild type (Student's *t* test). *, Whole seedling.

Growth Medium	Shoot DW/Seedling								
	Wild Type	*atptr1-1*	*atptr1-2*	*atptr5-1*	*atptr5-2*	*atptr1-1 atptr5-1*	*atptr1-2 atptr5-1*	35S:*PTR5*a	35S:*PTR5*b
					μg				
AM	555 ± 34	539 ± 71	569 ± 14	641 ± 43	493 ± 28	539 ± 17	509 ± 6	574 ± 17	645 ± 14
−N	55 ± 3	53 ± 1	49 ± 1	52 ± 2	44 ± 1	44 ± 3	55 ± 2	49 ± 1	45 ± 1
NH_4NO_3	79 ± 7	67 ± 7	73 ± 5	65 ± 3	63 ± 13	75 ± 5	66 ± 13	79 ± 9	79 ± 16
−N + peptide mix	149 ± 8	86 ± 4[a]	92 ± 2[a]	139 ± 6	136 ± 3	74 ± 2[a]	78 ± 5[a]	228 ± 12[a]	231 ± 15[a]
NH_4NO_3 + peptide mix	169 ± 22	103 ± 7[a]	103 ± 10[b]	151 ± 12	135 ± 10	101 ± 6[a]	101 ± 7[b]	267 ± 19[b]	259 ± 16[b]
−N + Ala-Ala	245 ± 17	109 ± 8[a]	129 ± 5[a]	194 ± 2	208 ± 13	115 ± 4[a]	107 ± 1[a]	561 ± 27[a]	518 ± 19[a]
−N + Pro-Ala	186 ± 8	100 ± 9[a]	108 ± 2[a]	152 ± 14	155 ± 16	79 ± 2[a]	95 ± 5[a]	498 ± 40[a]	447 ± 39[a]
−N + Ala-Phe	147 ± 6	125 ± 8	151 ± 4	128 ± 1	109 ± 14	148 ± 4	137 ± 3	40 ± 3*[b]	39 ± 0.1*[b]
−N + Ala + Pro	309 ± 17	352 ± 19	326 ± 21	347 ± 3	317 ± 17	361 ± 23	281 ± 67	378 ± 26	361 ± 14

[a] $P < 0.005$. [b] $P < 0.05$.

mutants and *35S:AtPTR5* lines did not reveal differences to wild-type plants when cultured on amino acids (Figs. 8 and 10; Table I). It remains to be investigated how much AtPTR1 contributes to N acquisition from the organic N pool of soil as opposed to acquisition of peptides retrieved from senescing root cells. Taken together, our results provide evidence that AtPTR1 has a crucial role in uptake of peptides into roots and that increased expression of peptide transporters can enhance uptake of N and plant growth.

CONCLUSION

Our study shows that AtPTR transporters have different roles within plants. AtPTR5 supplies germinating pollen with peptides and most likely functions in supplying peptides to maturating pollen, developing ovules, and seeds. In contrast, AtPTR1 mediates uptake of dipeptides into root cells. Overexpression of *AtPTR5* resulted in enhanced growth; thus, the ability to transport peptides across the plasma membrane appears to be generic and regulated primarily via tissue-specific expression. Further, our study provides novel evidence that the use of organic forms of N is not restricted to amino acids, but that di- and tripeptides should be considered as a potentially important N source as well.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Seeds of the Arabidopsis (*Arabidopsis thaliana* ecotype Columbia [Col-0]) mutant lines *atptr1-1*, *atptr1-2*, and *atptr5-1* were obtained from the SALK collection (Alonso et al., 2003; stock numbers Salk_131550, Salk_130803, and Salk_008661 respectively). The *atptr5-2* mutant line was obtained from the Wisconsin DsLox transposon-tagged lines (Woody et al., 2007, line WiscDsLox342H06). The locations of the T-DNA insertion sites were verified by sequencing PCR products amplified by using T-DNA left-border primers (for SALK lines, 5′-GCGTGGACCGCTTGCTGCAACT-3′; for Wisconsin line, 5′-AACGTCCGCAATGTGTTATTAAGTTGTC-3′) and gene-specific primers (for *atptr1* lines, 5′-AACACACCAACAACAACTAGACCACATAC-3′; for *atptr5* lines, 5′-ATATCAGCTTCGGTTCCTGGTCTAACAC-3′ and 5′-ATCAGAATTCGTGATTGCAGCCATC-3′). Lines were back-crossed twice with the wild type (Col-0) and selfed to isolate homozygous lines. Double knockout lines were isolated by crossing homozygous single *atptr1* mutants with the *atptr5-1* line; homozygous double mutants were identified in the BC2 progeny. The homozygous genotype of the insertion lines was confirmed by PCR on genomic DNA. All knockout lines were analyzed by RT-PCR for the absence of mRNA of *AtPTR1* (leaf tissue), and *AtPTR5* (flower tissue) or both transcripts as described below.

Arabidopsis ecotype Col-0 and mutant lines were grown in soil in a growth chamber at 22°C/18°C, 65% humidity, and 16 h of light (240 μmol m^{-2} s^{-1}). Plants were transformed by *Agrobacterium tumefaciens* (GV3101 pMP90) me-

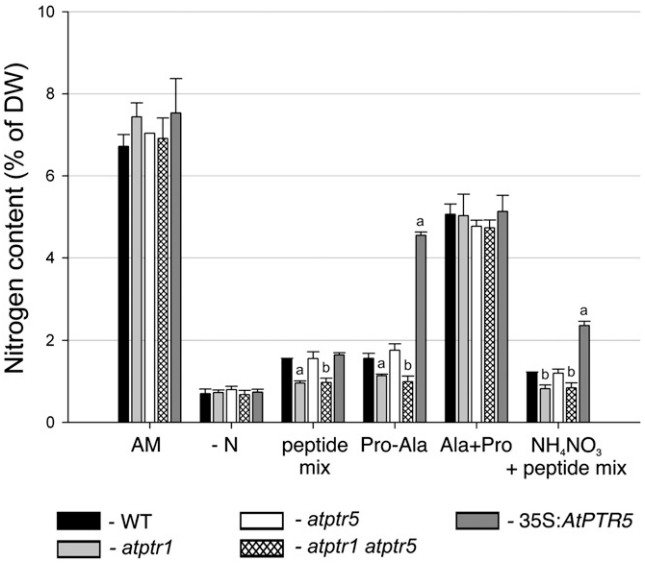

Figure 10. N content of wild-type (WT), *atptr1*, *atptr5*, *atptr1 atptr5*, and 35S:*AtPTR5* seedlings on medium containing different N sources. Seeds were germinated and cultivated for 2 weeks on different media as described in the Figure 8 and 9 legends. Data were obtained from three independent experiments. Values are the means of N ±SD (% of dry weight [DW]). Different letters indicate statistically significant differences compared to wild type (Student's *t* test). a, $P < 0.005$; b, $P < 0.05$.

diated gene transfer (Koncz and Schell, 1986) using the floral-dip method (Clough and Bent, 1998) as described earlier (Grallath et al., 2005).

For axenic culture, sterilized Arabidopsis seeds were vernalized in 0.1% agarose at 4°C in the dark for 2 d. Seeds were germinated and cultivated on either of the following media: (1) AM medium (2.16 g L^{-1} Murashige and Skoog salts [Duchefa]); (2) one-half-strength Murashige and Skoog medium (Murashige and Skoog, 1962) lacking NH_4NO_3 and KNO_3 replaced by KCl (−N; replacing KNO_3 by KCl did not have any effect on plant growth [e.g. biomass]); (3) −N medium containing 20 mM of a dipeptide as a sole nitrogen source; (4) −N medium containing 2.5 mM of an equimolar peptide mixture (Ala-Ala, Pro-Ala, Ala-Asp, Arg-Glu, and Ala-Gly); (5) −N containing 0.1 mM NH_4NO_3 and the peptide mixture. As a control, plants were cultivated on −N medium containing a mixture of the amino acids Ala and Pro (10 mM each). All media were adjusted to pH 5.8, completed with 1% Suc, and solidified with Oxoid agar (Oxoid). Seventy-five milliliters of medium was used per 12- × 12-cm plate and six lines, approximately 42 seeds each, were cultivated per plate. Alternatively, four lines and approximately 11 seeds per line were cultivated on 10 mL of medium (plate diameter 5 cm). Plants were cultivated for 2 weeks in a growth chamber under the above-mentioned conditions, except that the light intensity was 140 μmol m^{-2} s^{-1}.

For root growth experiments, seedlings were grown (vertically) on AM medium for 7 d. Sterile filter paper stripes soaked with water as a control or with a solution containing 1.1 μmol Ala-Eth were placed in front of the growing roots. The bottom of the plates was covered with aluminum foil and the plates were cultivated for another 10 d.

In Vitro Pollen Germination

For each genotype, pollen from three flowers was cultured in suspended drops in either GM as described by Footitt et al. (2007) or in GM containing 20 or 100 μM Ala-Eth (JPT Peptide Technologies). Germination was scored by microscopic examination and the germination rate was calculated from three independent experiments. Germinated pollen was visualized with a Zeiss Axioskop 2 microscope equipped with an Axiocam camera (Zeiss).

DNA Work

Standard methods (PCR, cloning procedure, transformation of bacteria, plasmid preparation, and DNA cleavage with restriction enzymes) were performed as described by Sambrook et al. (1989) and Ausubel et al. (1994).

AtPTR5-cDNA was isolated by RT-PCR using primer 5′-TCACACTTAAACTTGTCGAAACCAAAC-3′, primer 5′-TCAAAGCGCATGCCCGGTCGT-3′, and RNA extracted from flowers of Arabidopsis ecotype Col-0 as template. The *AtPTR5*-cDNA was cloned in the *Sma*I site of pDR196 (Rentsch et al., 1995) and verified by sequencing.

For translational fusions with GFP, the ORF of the *AtPTR5* cDNA was amplified by PCR and cloned in pUC18-spGFP6 and pUC18-GFP5Tsp (M. Suter-Grotemeyer and D. Rentsch, unpublished data). AtPTR5-GFP fusion, 5′-CTAGCTAGCATGGAAGATGACAAGGATATATACAC-3′, 5′-AATAGATCTTCAAGCGCATGCCCGGTCGTTTTC-3′ (ORF cloned into *Spe*I/*Bgl*II site). GFP-AtPTR5 fusion, 5′-CTAGCTAGCATGCGAAGATGACAAGGATATATACAC-3′, 5′-AATGTCGACTCAAAGCGCATGCCCGGTCG-3′ (ORF cloned into *Nhe*I/*Sal*I site). Sequence identity of all PCR-amplified fragments was verified by sequencing. For expression of the fusion proteins in yeast, the fragments were transferred to pDR-spGFP6 and pDR-GFP5-sp (M. Suter-Grotemeyer and D. Rentsch, unpublished data).

The *AtPTR5*-promoter region (2,582 bp upstream of the start codon) was amplified by PCR using genomic DNA of Arabidopsis ecotype Col-0 and primers 5′-ACCTACAAGGGACAATTGATTTTGG-3′ and 5′-TGTTGTTTGGTTTCGACAAGTTTAAGT-3′. The PCR product was cloned into the blunted *Spe*I site of pBluescript SK− (Stratagene) and sequenced. For plant transformation, the promoter was excised with *Bam*HI and *Not*I and transferred into the *Sma*I site of the binary vector pCB308 (Xiang et al., 1999). For overexpression in Arabidopsis, *AtPTR5* was used to circumvent cosuppression of *AtPTR1* in roots. The *AtPTR5*-cDNA was excised from pDR-*AtPTR5* with *Bam*HI and *Sal*I and cloned into corresponding sites of the binary vector pBinAR (Röber et al., 1996) downstream of the cauliflower mosaic virus 35S promoter (Gatz et al., 1992).

RNA Work

To quantify expression, total RNA was extracted using a method based on phenol-SDS extraction (Ausubel et al., 1994) including an additional DNase I treatment. RT was performed using the Moloney murine leukemia virus reverse transcriptase (USB) according to the manufacturer's instructions with oligo(dT) primers and 1.5 μg of total RNA as template, followed by PCR amplification of the 3′-cDNA region using cDNA-specific primers (for *AtPTR1*, 5′-AACACACCAACAACAACTAGACCACATAC-3′ and 5′-GTCAGGCTTGATTATGTC-3′; for *AtPTR5*, 5′-TGAGGAGTCTCTGCTCGG-3′ and 5′-ATCAGAATTCGTGATTGCAGCCATC-3′). For amplification of the region upstream of the insertion, the following primers were used: 5′-TTGTGCACACAGATAACTTAAAGTTT-3′ and 5′-TTGCCAGCGGGATAATGAACT-3′ (for *AtPTR1*), and 5′-GCTTCGGTTCCTGGTCTAACACCAAC-3′ and 5′-CAAACGAGAACAGAGGAAGCAATCA-3′ (for *AtPTR5*). The Arabidopsis actin gene *AtACT2* was used as a reference (5′-ATTCAGATGCCCAGAAGTCTTGTT-3′ and 5′-GAAACATTTTCTGTGAACGATTCCT-3′).

Northern-blot analyses were performed as described in Ausubel et al. (1994) according to the formaldehyde/formamide protocol using 15 μg of total RNA isolated from leaves and the cDNA of *AtPTR5* as a probe.

Determination of Peptide Concentration and Total N Content

Concentration of peptides and amino acids in the medium was determined by HPLC at ARS, University of Bern (Bidlingmeyer et al., 1984). Total N content of shoots was determined at the Washington State University Analytical Core laboratory; shoot material (1.5 mg) was combusted in a Costech ECS 4010 elemental analyzer, the resulting N_2 separated by gas chromatography and then analyzed using a Micromass Isoprime isotope ratio mass spectrometer (IRMS).

Yeast Growth, Transformation, and Selection

Saccharomyces cerevisiae strain LR2 (*MAT*α *hip1-614 his4-401 can1 ino1 ura3-52 ptr2*Δ*::hisG*; Rentsch et al., 1995) was transformed according to Dohmen et al. (1991), and transformants were selected on SC medium supplemented with 20 mM of His. To test for peptide transport activity, transformants were selected on SC medium containing 1 mM His-Ala as sole source of the required amino acid His (Rentsch et al., 1995).

Transient Expression in Protoplasts

Transient expression of GFP fusion proteins in tobacco (*Nicotiana tabacum*) protoplasts was performed as described (Dietrich et al., 2004). Samples were examined with a SP2 AOBS confocal microscope (Leica Microsystems). Filter settings were 500 to 520 nm for GFP and 628 to 768 nm for chlorophyll epifluorescence detection.

Staining for GUS Activity

Histochemical localization of GUS activity was performed as described previously (Dietrich et al., 2004). Pictures were taken with a Leica MZFLIII imaging system (Leica Microsystems) or a Zeiss Axioskop 2 microscope equipped with an Axiocam camera (Zeiss).

Expression in *Xenopus* Oocytes

Expression of AtPTR5 in *Xenopus* oocytes was performed as described in Meyer et al. (2006). Substrate-induced currents were measured 2 to 4 d after injection. The voltage protocol was as described in Dietrich et al. (2004), with the following modification: test potential (V_m) between +20 to −140 mV. Each voltage pulse was applied for 150 ms and the currents were filtered at 100 Hz.

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