Aromatic dipeptide Trp–Ala can be transported by Arabidopsis peptide transporters AtPTR1 and AtPTR5

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ABSTRACT
Dipeptides with an aromatic residue at the N–terminal position induced lower inward currents or blocked leak currents in Xenopus oocytes expressing the proton-coupled peptide transporter AtPTR1 or AtPTR5 of Arabidopsis thaliana compared with dipeptides with an aromatic residue at the C–terminal position. Here, AtPTR1 and AtPTR5 were expressed in a yeast mutant of peptide transporter (ptr2) with tryptophan auxotrophy. Growth assays showed that Trp–Ala could be transported by both AtPTR1 and AtPTR5 as efficiently as Ala–Trp. Our data suggested that the previous finding in Xenopus oocytes might be an artifact of heterologous expression, and that AtPTR1 and AtPTR5 expressed in yeast could transport dipeptides with an aromatic residue at the N–terminal position.

KEYWORDS
aromatic; dipeptide; NPF; peptide transporter; PTR; small peptide

Introduction

Amino acids are the major form of organic nitrogen (N) in plants and the fundamental elements of proteins. Proteolysis occurs ubiquitously in different organelles, cells, and organs throughout the life cycle of plants. This process plays important roles in protein turnover and reallocation of organic N, particularly during germination and senescence. Amino acids, as well as small peptides, are commonly considered products of proteolysis. In germinating barley seeds, in which intensive proteolysis is activated to fulfill the rapid translocation of storage protein from the endosperm to embryos, the pool size of peptides is comparable to that of free amino acids.1 Furthermore, peptides can be absorbed by germinating barley embryos at higher rates compared with free amino acids.2 Physiological studies with germinating barley embryos have indicated the existence of a single common proton–coupled active carrier system for short peptides (2–5 residues).3,4 Many peptide transporters have been characterized in the past 2 decades, providing a molecular view of the peptide transport system.5–7

Peptide transporters of the PTR/POT/NPF family, which transport di-/tripeptides with the proton gradient, exhibit enigmatic substrate multispecificity.8–10 The multispecificity makes it possible for a single peptide transporter to recognize 400 unmodified dipeptides and 8,000 unmodified tripeptides as well as thousands of modified peptides and non–ribosomal–synthesized peptides.11

The plasma–membrane–localized peptide transporter of Arabidopsis thaliana, AtPTR1 (AtNPF8.1), functions in uptake of peptides by roots, while its homolog AtPTR5 (AtNPF8.2) plays a role in peptide transport into germinating pollen.12 The 2 peptide transporters share similar substrate multispecificity and electrophysiological properties. Unexpectedly, when these transporters were expressed in Xenopus laevis oocytes, alanine–containing dipeptides with an aromatic residue at the N–terminal position (Phe–Ala, Tyr–Ala, and Trp–Ala) induced lower inward currents or blocked leak currents compared with dipeptides with an aromatic residue at the C–terminal position.
(Ala–Phe, Ala–Tyr, and Ala–Trp). Therefore, the authors proposed that dipeptides with an aromatic residue at the N–terminal position might act as competitive inhibitors rather than as substrates of AtPTR1 and AtPTR5. Dipeptides with an aromatic residue at the N–terminal position are expected to be at low concentrations in plants because of the low frequencies of aromatic amino acids in proteins. Nevertheless, the high energy cost of the biosynthesis of aromatic amino acids enables plant cells to reutilize any peptides with an aromatic residue. In yeast, dipeptides containing an aromatic residue exhibit high affinities to the peptide transporter PTR2p, particularly with an aromatic residue at the N–terminal position. In other organisms, hPepT1, hPepT2, PepTst, and YjdL also show similar substrate preferences for PTR2p. Thus, dipeptides with an aromatic residue at the N–terminal position may be natural substrates of AtPTR1 and AtPTR5.

Materials and methods

Saccharomyces cerevisiae strain ABC738 (MATa ura3–52 leu2–Δ1 lys2–801 his3–Δ200 trpl–Δ63 ade2–101 ptr2Δ::KanMX2) was obtained from the Institute of Microbial Technology, India. The yeast expression vector pYES260 was kindly offered by Professor M. Zhang from South China Botanical Garden. Ala–Trp and Trp–Ala were synthesized by GL Biochem. Difco™ Yeast Nitrogen Base (YNB) without Amino Acids (Cat# 291920) was purchased from BD. Agar (Cat# A505255) was purchased from Sangon Biotech. Minimal Synthetic Dextrose (SD) Base (Cat# 630411), Drop-out (DO) Supplement -Trp/-Ura (Cat# 630427), and DO Supplement -Ura (Cat# 630416) were purchased from Clontech. Alanine (Cat# A7627), tryptophan (Cat# T0254), and galactose (Cat# V900922) were purchased from Sigma–Aldrich.

The coding DNA sequences (CDS) of AtPTR1 and AtPTR5 were amplified from leaf cDNA of Arabidopsis thaliana Col-0. The gene–specific primers were 5′–tcaGGTACCATAAGGAAGAGATGTGTGATTACG–3′ and 5′–cagTCTAGATCAATGTGCTCGACCAACAG–3′ for AtPTR1, and 5′–tcaGGTACCATAAGGAAGAGATGTGTGATTACG–3′ and 5′–cagTCTAGATCAATGTGCTCGACCAACAG–3′ for AtPTR5, with KpnI and XbaI sites (underlined). The PCR fragments were cloned into pYES260 with a modified multiple cloning site (MCS). The recombinant vectors, as well as the empty vector pYES260, were transformed into ABC738 according to Yeast Protocols Handbook (Clontech). The transformants were spread on -Ura selection plates (Minimal SD Base 26.7 g L⁻¹, DO Supplement -Ura 0.77 g L⁻¹, and agar 15 g L⁻¹, pH 5.5) and cultured at 30°C for 48 hours. The resulting transformants were ABC738–AtPTR1, ABC738–AtPTR5, and AB738-EV.

For cell growth analysis, transformants were pre-cultured in -Ura selection medium (Minimal SD Base 26.7 g L⁻¹, and DO Supplement -Ura 0.77 g L⁻¹, pH 5.5) at 30°C for 24 hours. Pre-cultures were washed with sterile water and diluted to OD₆₆₀ = 0.05 in each test medium (galactose 20 g L⁻¹, YNB 6.7 g L⁻¹, DO supplement -Trp/-Ura 0.72 g L⁻¹, pH 5.5, and supplemented with Ala–Trp 100 μM (AW), Trp–Ala 100 μM (WA) or no addition (-Trp)) and incubated at 30°C. Culture turbidity was monitored by measuring OD₆₆₀ using a spectrophotometer.

For spot assays, transformants were pre-cultured in -Ura selection medium (Minimal SD Base 26.7 g L⁻¹, and DO Supplement -Ura 0.77 g L⁻¹, pH 5.5) at 30°C for 24 hours. Pre-cultures were washed with sterile water and diluted to OD₆₆₀ = 4.0, 0.8, 0.16, and 0.03. Five μL cell suspensions were inoculated on plates with different tryptophan sources (agar 15 g L⁻¹, galactose 20 g L⁻¹, YNB 6.7 g L⁻¹, DO supplement -Trp/-Ura 0.72 g L⁻¹, pH 5.5, and supplemented with tryptophan + alanine (+Trp), Ala–Trp (AW), Trp–Ala (WA) or no addition (-Trp)) and incubated at 30°C for 48 hours or 4 d.

Results

To examine whether dipeptides with an aromatic residue at the N–terminal position can be transported by AtPTR1 and AtPTR5, these peptide transporters were expressed in a ptr2 yeast mutant strain ABC738 with tryptophan auxotrophy. If Trp–Ala could not be transported by these peptide transporters, the recombinant yeast strains expressing these peptide transporters (ABC738–AtPTR1 and ABC738–AtPTR5) could not grow in synthetic medium containing Trp–Ala as the sole tryptophan source. We first confirmed the tryptophan auxotrophy of the recombinant yeast strains. Neither ABC738–AtPTR1 nor ABC738–AtPTR5 grew in tryptophan–defected medium (-Trp) (Fig. 1 and Fig. 2), indicating that neither peptide
transporter altered the tryptophan auxotrophy of ABC738. ABC738–AtPTR1 or ABC738–AtPTR5 was inoculated in tryptophan–defected medium supplemented with 100 μM Ala–Trp (AW) or Trp–Ala (WA) as the sole tryptophan source. Either of these strains could grow and showed similar growth rates in both AW and WA media (Fig. 1). The recombinant strains with the empty vector (ABC738–EV) did not

Figure 1. Trp–Ala was transported by AtPTR1 and AtPTR5. Culture turbidity of S. cerevisiae strains ABC738–AtPTR1 (A) and ABC738–AtPTR5 (B), representing the parental ABC738 strain transformed with constructs encoding Arabidopsis AtPTR1 and AtPTR5, respectively, was monitored by OD_{600} in each test media (galactose 20 g L⁻¹, YNB 6.7 g L⁻¹, DO supplement -Trp/-Ura 0.72 g L⁻¹, pH 5.5, and supplemented with Ala–Trp 100 μM (AW), Trp–Ala 100 μM (WA) or no addition (-Trp)) at 30 °C. For either cell strain, data from a single transformant were present, and 3 transformants from 2 experiments showed similar results.

Figure 2. Trp–Ala was transported with a similar efficiency to Ala-Trp. Spot assays on agar plates with a range of concentrations of tryptophan sources. Yeast cells of ABC738–AtPTR1, ABC738–AtPTR5, and ABC738–EV, representing respectively the parental ABC738 strain transformed with constructs encoding Arabidopsis AtPTR1 and AtPTR5 and with the empty vector, were cultured on agar plates with different tryptophan sources (agar 15 g L⁻¹, galactose 20 g L⁻¹, YNB 6.7 g L⁻¹, DO supplement -Trp/-Ura 0.72 g L⁻¹, pH 5.5, and supplemented with tryptophan + alanine (+Trp), Ala–Trp (AW), Trp–Ala (WA) or no addition (-Trp)) at 30 °C for 48 h (A) and 4 d (B). The concentrations of tryptophan sources were 100 μM (A), 50 μM, 5 μM, and 1 μM (B). Alanine was added at the same concentrations as tryptophan in (+Trp) media. A separate assay showed similar results.
grow in these media, suggesting that the growth of ABC738-AtPTR1 and ABC738-AtPTR5 in the AW and WA media was not related to the existence of free tryptophan contamination (Fig. 2). In spot assays with a range of concentrations of Ala-Trp and Trp-Ala as the sole tryptophan sources, ABC738-AtPTR1 and ABC738-AtPTR5 grew even in 1 μM of Trp-Ala and showed similar growth in the AW and WA media at all concentrations tested (Fig. 2). Therefore, these data demonstrated that Trp–Ala was transported by AtPTR1 and AtPTR5 as efficiently as Ala–Trp to support the growth of the tryptophan auxotrophic ABC738.

**Discussion**

Proton–coupled peptide transporters (POT/PTR/SCL15) are thought to function in the uptake of di-/tripptides for amino acid recycling. Here, we confirmed that Trp–Ala could be transported by the *Arabidopsis* peptide transporters AtPTR1 and AtPTR5 in yeast (Fig. 1) and was not a competitive inhibitor. In addition, several dipeptides with an aromatic residue at the N-terminal position (Phe–Ala, Phe–Phe, Trp–Ala, Trp–Trp, and Tyr–Ala) could be transported by mammalian peptide transporters PepT1 and PepT2 expressed in *Xenopus* oocytes. However, when the *Arabidopsis* peptide transporters AtPTR1 and AtPTR5 were expressed in *Xenopus* oocytes, Trp–Ala, not Ala–Trp, blocked the leak current for AtPTR1 and induced a much smaller inward current for AtPTR5. Other dipeptides with an aromatic residue at the N-terminal position (Tyr–Ala, Phe–Ala, and Phe–Phe) exhibited similar properties to these peptide transporters. Because Trp–Ala is predominantly a zwitterion at either pH 5.5 or pH 7.5, proton–coupled transport of Trp–Ala must be electrogenic, regardless of the stoichiometry of proton and Trp-Ala. Regarding the data for *Xenopus* oocyte expression, (i) Trp–Ala may act as a competitive inhibitor because of the malfunction of the heterologously expressed peptide transporters or (ii) Trp–Ala may be transported by these peptide transporters in a proton/cation–independent manner. An uptake assay of isotope-labeled Trp–Ala should be conducted to clarify whether this dipeptide can be transported into *Xenopus* oocytes by these *Arabidopsis* peptide transporters. If Trp–Ala can be transported into *Xenopus* oocytes, it implies that AtPTR1 and AtPTR5 transport dipeptides with an aromatic residue at the N-terminal position in a proton/cation–independent manner, at least in the context of heterologous expression in *Xenopus* oocytes. Given that peptidase activities are sufficient to hydrolyze absorbed peptides in *Xenopus* oocytes as well as in plant cells, a concentration gradient is expected for facilitated diffusion of such dipeptides. Similar to PepT2, AtPTR1 and AtPTR5 showed a substrate–independent leak current, which could be blocked by extracellular Trp–Ala and Phe–Phe. Moreover, the substitution mutant R282E–rbPepT1 appeared to have a peptide–gated cation conductance and facilitated diffusion pathway for peptides, while the wild-type of this intestinal low-affinity peptide transporter co–transported peptides and proton obligatorily.

Although these data provide some support for the possibility that AtPTR1 and AtPTR5 transport dipeptides with an aromatic residue at the N-terminal position in a proton/cation–independent manner, this remains controversial, as these dipeptides are proton–coupled transported by the mammalian homologs PepT1 and PepT2. Therefore, proteoliposome assays monitoring both peptide and proton flux with *in planta*-expressed peptide transporters may clarify these results.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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**Author contributions**

DQ and MZ designed the experiments. DQ, RH, and YL performed the experiments and analyzed the data. DQ, RH and MZ wrote the manuscript.

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