Transport Function of Rice Amino Acid Permeases (AAPs)

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The transport function of four rice (Oryza sativa) amino acid permeases (AAPs), OsAAP1 (Os07g04180), OsAAP3 (Os06g36180), OsAAP7 (Os05g34980) and OsAAP16 (Os12g08090), was analyzed by expression in Xenopus laevis oocytes and electrophysiology. OsAAP1, OsAAP7 and OsAAP16 functioned, similarly to Arabidopsis AAPs, as general amino acid permeases. OsAAP3 had a distinct substrate specificity compared with other rice or Arabidopsis AAPs. OsAAP3 transported the basic amino acids lysine and arginine well but selected against aromatic amino acids. The transport of basic amino acids was further analyzed for OsAAP1 and OsAAP3, and the results support the transport of both neutral and positively charged forms of basic amino acids by the rice AAPs. Cellular localization using the tandem enhanced green fluorescent protein (EGFP)–red fluorescent protein (RFP) reporter pHusion showed that OsAAP1 and OsAAP3 localized to the plasma membrane after transient expression in onion epidermal cells or stable expression in Arabidopsis.

Keywords: amino acid transporters ● electrophysiology ● rice AAPs ● Xenopus oocytes.

Abbreviations: AAP, amino acid permease; CaMV, Cauliflower mosaic virus; EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; GFP, green fluorescent protein; MS, Murashige and Skoog; RFP, red fluorescent protein; TMS, transmembrane span.

Introduction

Plants utilize several different families of transporters for amino acid uptake. These are amino acid permeases (AAPs), lysine-histidine-like transporters (LHTs), proline transporters (ProTs), γ-aminobutyric acid transporters (GATs), ANT1-like aromatic and neutral amino acid transporters and cationic amino acid transporters (CATs) (Wipf et al. 2002, Rentsch et al. 2007). Here, we investigated the transport activity of four AAPs from rice (Oryza sativa). In Arabidopsis, AAPs are important for phloem transport of amino acids (Hunt et al. 2010, Zhang et al. 2010). Although the biochemical transport function of AAPs has not been previously studied in monocots, AAPs may also function in long-distance transport of amino acids in monocots. A wide range of amino acids is present in the phloem sap of rice (Fukumorita and Chino 1982, Hayashi and Chino 1990, Hayashi et al. 1993). This is consistent with the wide substrate specificity of AAPs in Arabidopsis (Fischer et al. 1995, Fischer et al. 2002). In Arabidopsis and legumes, AAPs are also important for amino acid uptake into seeds, as reviewed by Tegeder (2014). This also appears to be true for rice since OsAAP6 (Os01g65670) is expressed in seeds and was identified as underlying a positive quantitative trait locus (QTL) for grain protein content (Peng et al. 2014).

AAPs are a plant-specific family but belong to the larger amino acid/auxin permease superfamily (AAAP) (Rentsch et al. 2007) with representatives in animals and fungi. AAPs were first discovered in Arabidopsis by complementation of yeast strains deficient in amino acid uptake (Frommer et al. 1993, Hsu et al. 1993, Fischer et al. 1995). Arabidopsis encodes eight AAPs, and all except AtAAP7 have been functionally characterized. AAPs co-transport protons and amino acids into cells at a ratio of 1:1 (Boorer et al. 1996, Boorer and Fischer 1997). Hydropathy analysis predicts the AAPs to have from 10 to 12 transmembrane spans (TMS) (Hsu et al. 1993) and AtAAP1 was experimentally demonstrated to have 11 TMS with the N-terminus on the cytoplasmic side (Chang and Bush 1997). The Arabidopsis AAPs transport amino acids with a broad specificity and moderate affinity (Fischer et al. 2002). Amino acid charge appears to play a role in AAP substrate specificity. AtAAPs generally do not transport aspartate, except for AtAAP6 and AtAAP8 (Okumoto et al. 2002), and only AtAAP3 and AtAAP5 can transport the basic amino acids lysine and arginine at a high rate (relative to alanine) (Fischer et al. 2002).

Arabidopsis AAP genes have distinct expression patterns, suggesting functional specialization. AtAAP1, AtAAP2 and AtAAP8 are expressed in siliques and seeds (Hirner et al. 1998, Okumoto et al. 2002), AtAAP6 is expressed in xylem parenchyma (Okumoto et al. 2002) and AtAAPs is expressed throughout the plant (Fischer et al. 1995). AtAAP2 is expressed in the phloem (Zhang et al. 2010). Mutant analysis indicates roles for the AtAAPs in long-distance amino acid transport, especially in phloem loading and the transport of amino acids into seed endosperm. Arabidopsis ataat8 mutants have an approximately 50% reduction in seed set (Schmidt et al. 2007), and ataat1 mutants have altered amino acid composition in the seeds (Sanders et al. 2009). ataat6 mutants have altered phloem amino acid composition (Hunt et al. 2010). In addition, ataat1 and ataat6 mutants have increased resistance to toxic levels of amino acids in plant medium, suggesting a possible role for these AAPs in root amino acid uptake (Lee et al. 2007, Svennerstam et al. 2008).

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AAPs have been studied in a variety of eudicot species. Localization studies in legumes suggest a role for AAPs in transport of amino acids into seeds in pea (Tegeder et al. 2000) and broad bean (Miranda et al. 2001) as well as xylem to phloem transfer in French bean (Tan et al. 2008). StAAP1 in potato is expressed in leaves and plays a possible role in phloem loading (Koch et al. 2003). Expression of NaAAP1 was detected in the bundle sheath cells surrounding vascular bundles in pitchers of *Nepenthes alata*, a pitcher plant (Schulze et al. 1999). PtAAP11 is expressed in poplar in differentiating xylem cells and has a high affinity for amino acids (Couturier et al. 2010). Therefore, analysis of AAP expression in eudicots suggests a predominant role for these transporters in vascular tissue.

The transport function of AAPs in monocots has not been previously studied. Nineteen AAP genes were identified in rice (Tegeder and Ward 2012, Zhao et al. 2012), more than twice as many as in Arabidopsis. Here, we use the gene nomenclature for rice AAPs proposed by Zhao et al. (2012). Phylogenetic analysis shows that several clades contain sequences from rice not present in Arabidopsis (Tegeder and Ward 2012). In this report we cloned and analyzed the transport activity of OsAAP1 (Os07g04180), OsAAP7 (Os05g34980), OsAAP16 (Os12g08090) and OsAAP3 (Os06g36180).

**Results**

RNA was extracted from leaves of 2-week-old and 6-week-old rice plants and used for cDNA synthesis. Four OsAAP coding sequences were cloned into the oocyte expression vector pOO2/GW (Sun et al. 2010). The OsAAP proteins are 475–496 amino acids long, similar in length to Arabidopsis AAPs. By phylogenetic analysis OsAAP1 clusters with AtAAP1, AtAAP6 and AtAAP8 from Arabidopsis (Fig. 1; Tegeder and Ward 2012), therefore, it is a monocot representative of this well-studied clade. OsAAP3 has two close homologs in rice (Fig. 1) but none from Arabidopsis or *Medicago truncatula*. OsAAP7 and OsAAP16 are found in a monocot-specific clade containing seven AAPs from rice (Fig 1; Tegeder and Ward 2012), and no transporters have been functionally characterized from this clade.

To study the transport function of the rice AAPs, each was expressed in *Xenopus laevis* oocytes, and two-electrode voltage clamping was used to record amino acid-dependent currents. Oocytes were perfused with Na Ringer at pH 5.6 and voltage-clamped at –40 mV. All four rice AAPs showed large amino acid-induced inward currents. The amount of cRNA injected was adjusted so that similar current levels were obtained for each transporter. Representative currents are shown for OsAAP3 and OsAAP7 (Fig. 2). When amino acids (10 mM) were applied in the bath solution, inward currents (downward deflections) were measured, consistent with proton-coupled transporter activity (Fig. 2B, C). When amino acids were removed from the bath solution, currents returned to baseline. Uninjected oocytes showed no changes in current upon application of amino acids (Fig. 2A). Both OsAAP3 and OsAAP7 showed broad substrate specificity. Both transported glycine, phenylalanine and serine, but showed low or undetectable current with β-alanine and aspartate (Fig. 2B, C).

Currents were recorded at membrane potentials from 57 to –138 mV in the absence and presence of amino acids.
Background currents (Fig. 3A, C, squares) were subtracted from currents in the presence of 10 mM glycine (Fig. 3A, C, triangles) to obtain substrate-dependent currents (Fig. 3B, D). Currents induced by 10 mM glycine for OsAAP7 are shown as an example (Fig. 3A, B), and similar results were obtained for OsAAP1 and OsAAP16. The current–voltage relationship for OsAAP3 was different. Instead of inward currents increasing linearly at negative potentials in the range of –60 to –137 mV as seen for OsAAP7 (Fig. 3B), currents saturated at negative potentials for glycine (Fig. 3D) as well as for asparagine, cysteine, glutamate, proline, phenylalanine and tyrosine (not shown).

Substrate-dependent currents induced by 10 mM of each of the 20 proteinogenic amino acids and β-alanine were measured (Fig. 4). Substrate-induced currents were normalized to currents induced by 10 mM glycine to control for differences in expression level between oocytes. OsAAP1, OsAAP7 and OsAAP16 showed similar substrate specificities with one exception: arginine was transported well by OsAAP7 and OsAAP16, but at only 6% of the glycine-induced current in OsAAP1-expressing oocytes (Fig. 4). However, OsAAP3 showed a different substrate specificity. The aromatic amino acids phenylalanine, tyrosine and tryptophan, as well as valine and isoleucine, induced less current in OsAAP3-expressing oocytes compared with oocytes expressing the other OsAAPs, and currents induced by tryptophan were not detectable.

Basic amino acids were transported relatively well by OsAAP3 (Fig. 4). We compared the affinity of OsAAP1, as a representative of well-characterized AAPs, with that of OsAAP3 for positively charged amino acids and uncharged amino acids of similar size. Histidine (pKₐ 6.00), lysine (pKₐ 10.53) and arginine (pKₐ 12.48) carry a net positive charge at the pH of the bathing solution (pH 5.6). Two additional substrates, methionine and citrulline, have similar sized side chains to lysine and arginine, respectively, but do not carry a net charge at pH 5.6. Non-linear regression using the Michaelis–Menten equation was performed on each concentration–activity curve to calculate Kₐ₅₀. OsAAP3 displayed similar Kₐ₅₀ values for lysine and methionine, as well as arginine and citrulline (Fig. 5). If OsAAP3 only transported the uncharged form of amino acids we would expect the apparent Kₐ₅₀ to be much higher for arginine and lysine because only a small fraction of these basic amino acids is present in the uncharged form at the acidic pH of the bath solution. While it is clear that OsAAP3 transports uncharged amino acids in symport with protons, the results indicate that, in addition, OsAAP3 transports the positively charged form of
lysine and arginine. OsAAP1 showed a higher affinity for methionine ($K_{0.5} = 0.57 \text{ mM}$) compared with lysine ($K_{0.5} = 27 \text{ mM}$) and a higher affinity for citrulline ($K_{0.5} = 1.8 \text{ mM}$) compared with arginine ($K_{0.5} > 100 \text{ mM}$). At pH 5.6, when the total lysine concentration is 27 mM (the measured $K_{0.5}$ of OsAAP1), the concentration of uncharged lysine is 0.317 mM, orders of magnitude lower than $K_{0.5}$ values measured for other amino acid substrates. Therefore, we conclude that both OsAAP1 and OsAAP3 transport the charged forms of basic amino acids. These transporters are also likely to transport uncharged forms of lysine and arginine as well, based on their ability to transport methionine and citrulline.

AAP proteins have 11 TMS with the N-terminus in the cytosol and the C-terminus in the apoplast. N-terminal green fluorescent protein (GFP) fusions have been used with AtAAP1 to demonstrate plasma membrane localization (Lee et al. 2007). However, in our study, N-terminal fusion of GFP with OsAAP1, OsAAP3, OsAAP7 and OsAAP16 interfered with expression in onion epidermal cells, resulting in low expression that appeared punctate (not shown). A C-terminal GFP fusion to OsAAP7 resulted in endoplasmic reticulum (ER)-localized fluorescence (not shown). This is expected since the C-terminus is located in the ER lumen/apoplastic side of the membrane. GFP is quenched at the acidic pH expected in the apoplast and the apparent ER localization reflects the portion of the AAP protein present in the ER.

To obtain more information on cellular localization of the rice AAPs, C-terminal fusions of the pH reporter pHusion, a tandem enhanced green fluorescent protein (EGFP)–red fluorescent protein (RFP) construct, were used (Gjetting et al. 2012). RFP is relatively stable at low pH, allowing pHusion to be used as a ratiometric probe for cellular pH (Gjetting et al. 2012). Here, we used pHusion to differentiate between localization of the C-termini of OsAAP1 and OsAAP3 in the ER lumen vs. the apoplast (Fig. 6) to determine whether these transporters are localized to the plasma membrane. AAP localization was analyzed using both transient expression in onion epidermis and stable expression in Arabidopsis. For OsAAP1, GFP and RFP fluorescence were visible throughout the ER in onion cells (Fig. 6A, B) and in Arabidopsis root cells (Fig. 6D, E). In the overlay images, RFP fluorescence was visible around the perimeter of the cell where GFP was not seen (Fig. 6C, F). This is consistent with a localization of the C-terminus of OsAAP1 in the acidic apoplast where RFP fluorescence but not GFP fluorescence can be observed. Similar results were obtained for OsAAP3 in both onion epidermal cells (Fig. 6G–I) and Arabidopsis roots (Fig. 6J–L) where GFP and RFP fluorescence appear predominantly cytoplasmic, but in the overlays RFP can be seen at the cell periphery. Therefore, we conclude that OsAAP3 and OsAAP1 are located in the plasma membrane. To rule out the possibility that red fluorescence around the cell is the result of autofluorescence of the cell wall, an onion epidermal cell not expressing a pHusion construct was imaged at the same laser intensity. No significant autofluorescence was found (not shown).

**Discussion**

Many more AAP genes are present in rice and other monocots compared with Arabidopsis (Fig. 1). Therefore, even though the function of AAPs in Arabidopsis has been studied, it is important to analyze the function of monocot AAPs directly. In fact, 11 AAPs encoded by rice occur in clades that do not contain AAPs from Arabidopsis or *M. truncatula* (Tegeder and Ward 2012). There may be some different transport activity or function of AAPs in monocots that has not been discovered in Arabidopsis.
Fig. 5 Kinetic analysis of methionine, lysine, citrulline, arginine and histidine transport by OsAAP1 and OsAAP3. Oocytes expressing OsAAPs were perfused with Na Ringer solution at pH 5.6 at a holding potential of –40 mV. Substrate-induced currents (background subtracted) were measured at –137.6 mV. Error bars represent the mean ± SE (n = 4 except for OsAAP1 lysine, citrulline and OsAAP3 lysine, histidine where n = 3). Currents were normalized to $V_{\text{max}}$. 

$K_{0.5}$ values for each substrate are as follows:

- **OsAAP1**
  - Methionine: $K_{0.5} = 0.570 ± 0.110 \text{ mM}$
  - Lysine: $K_{0.5} = 27.2 ± 6.80 \text{ mM}$
  - Citrulline: $K_{0.5} = 1.84 ± 0.401 \text{ mM}$
  - Arginine: $K_{0.5} = 126.75 ± 30.19 \text{ mM}$
  - Histidine: $K_{0.5} = 7.09 ± 1.76 \text{ mM}$

- **OsAAP3**
  - Methionine: $K_{0.5} = 2.24 ± 0.671 \text{ mM}$
  - Lysine: $K_{0.5} = 3.45 ± 0.856 \text{ mM}$
  - Citrulline: $K_{0.5} = 2.12 ± 0.867 \text{ mM}$
  - Arginine: $K_{0.5} = 1.02 ± 0.283 \text{ mM}$
  - Histidine: $K_{0.5} = 10.1 ± 3.94 \text{ mM}$
OsAAP1 belongs to a phylogenetic clade that also contains three previously characterized AAPs from Arabidopsis: AtAAP1, AtAAP6 and AtAAP8 (Tegeder and Ward 2012). We were interested to know how similar the activity of OsAAP1 was to the most related AAPs from Arabidopsis. OsAAP1 has a low affinity for basic amino acids similar to AtAAP1 and AtAAP6 (Fischer et al. 2002). AtAAP6 has a high affinity for histidine (Fischer et al. 2002) that is not shared by OsAAP1 or AtAAP1, indicating that substrate affinity is not very consistent within clades of AAPs. OsAAP7 and OsAAP16 belong to a monocot-specific clade, but their substrate specificity was quite similar to that of OsAAP1, with the exception that OsAAP7 and OsAAP16 transport arginine to a greater extent than OsAAP1. OsAAP1, OsAAP7 and OsAAP16 functioned as

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**Fig. 6** Plasma membrane localization of OsAAP1–pHusion and OsAAP3–pHusion in onion epidermal cells and Arabidopsis root cells. OsAAP1:pHusion or OsAAP3:pHusion were transiently expressed in onion epidermis by particle bombardment or expressed in Arabidopsis roots by stable transformation. (A, B, C) OsAAP1 in onion epidermis; (D, E, F) OsAAP1 in Arabidopsis roots; (G, H, I) OsAAP3 in onion epidermis; (J, K, L) OsAAP3 in Arabidopsis roots.
general AAphans and transported all amino acids well except asparagine and β-alanine. This is similar to the activity of most Arabidopsis AAphans. In Arabidopsis, only AtAAP6 transports asparagine well and β-alanine is only transported by AtAAP3 (Fischer et al. 2002).

OsAAP3 is in a clade with no clear representatives in Arabidopsis or M. truncatula, and the substrate specificity of OsAAP3 was quite different from that of all other AAphans characterized to date. OsAAP3 displayed low or undetectable transport of valine, tyrosine, tryptophan, phenylalanine and isoleucine. However, it transported alanine, leucine and methionine very well. OsAAP3 was also distinct from other rice AAphans we tested in that it transported the basic amino acids lysine and arginine very well (Fig. 4). Lysine and arginine also induce large currents in Xenopus oocytes expressing AtAAP3 and AtAAPs from Arabidopsis (Fischer et al. 2002).

The substrate specificity and H⁺:amino acid stoichiometry of Arabidopsis AtAAP1 and AtAAPs were studied in detail using a combination of electrophysiology and radiolabeled substrate uptake (Boorer and Fischer 1997). AtAAP3 transports the uncharged form of neutral and acidic amino acids and transports the positively charged form of basic amino acids (Boorer and Fischer 1997). The stoichiometry of AtAAP1 and AtAAP3 is fixed at 1:1 (H⁺:amino acid) regardless of amino acid charge. We addressed the question of whether rice AAphans transport the charged form of basic amino acids. At pH 5.6, 99.9988% of lysine and 99.999987% of arginine exists in the positively charged form. The relatively large currents induced by lysine and arginine in oocytes expressing OsAAP3 indicate that the positively charged form of basic amino acids was transported. OsAAP1 and OsAAP3 transport the uncharged amino acids methionine and citrulline; therefore, it is likely that the uncharged lysine and arginine are also transported but the rate is expected to be quite low at pH 5.6.

Expression of AAphans in rice has been studied using microarrays (Jain et al. 2007). OsAAP1 expression is low throughout the plant, with the highest expression in seeds, OsAAP3 is highly expressed in all organs, especially roots and inflorescences, OsAAP7 has high expression in roots and leaves, and OsAAP16 shows high expression in roots, leaves, flowers and seeds. Expression of about half of the 19 rice AAphan genes is regulated by stress (drought, salt or cold stress); however, the expression of the four rice AAphan genes analyzed herein is not regulated by stress (Jain et al. 2007). To understand the physiological role of each AAphan, higher resolution expression at the cell level will be required.

The use of fluorescent protein fusions to study subcellular localization is a challenge for the AAP family because they have an odd number of TMS. AtAAP1 was demonstrated to have 11 TMS (Chang and Bush 1997) and we expect that all AAphans have the same topology. Since GFP fluorescence is quenched at acidic pH, GFP fusions that position GFP either in acidic environments such as the vacuolar lumen or in the wall space may not provide an accurate localization of AAphan proteins. Previously, AtAAP1 (Lee et al. 2007) and AtAAP3 (Okumoto et al. 2004) have been localized to the plasma membrane using N-terminal GFP fusions. For the rice AAphans, N-terminal GFP fusions led to low expression or punctate cytoplasmic fluorescence, indicating that the GFP interfered with biosynthesis (not shown). Therefore, we used the EGF–RFP tandem fluorescent protein pHusion (Gjetting et al. 2012) fused to the C-terminal end of OsAAP1 and OsAAP3 to determine localization. The majority of EGF and RFP fluorescence was intracellular, consistent with localization in the ER. However, RFP but not EGF fluorescence was observed in the cell periphery, consistent with a plasma membrane localization (Fig. 6). The predominant ER localization could be due to the use of a strong promoter [2 x Cauliflower mosaic virus (CaMV) 35 S], but similar intracellular localization was also observed for AtAAP3 and was suggested to be due to trafficking or cycling of the transporter (Okumoto et al. 2004), which could also be true for the rice AAphants. A C-terminal fusion of GFP with rice OsAAP3 was localized to the ER (Peng et al. 2014). Based on the topology of AAphans and results reported here, we suggest that it is more likely that OsAAP6 is a plasma membrane protein and that fluorescence observed in the ER was due to protein during biosynthesis or trafficking. Our results using pHusion are consistent with a plasma membrane localization for rice AAphans.

Materials and Methods

Phylogenetic analysis

Full-length protein sequences were aligned using Clustal X (Larkin et al. 2007). Maximum likelihood analysis was carried out using PhyML 3.0 with 100 bootstrap replicates (Guindon and Gascuel 2003, Guindon et al. 2010) through the iPlant Collaborative website (www.iplantcollaborative.org). Trees were visualized using the FigTree program (http://tree.bio.ed.ac.uk/software/figtree/).

Cloning of AAphants from rice

Rice plants (Oryza sativa var. Kitaake) were grown in Turface MVP (Turface Athletics) in 8 inch pots outdoors. RNA was extracted from leaves of 2-week-old and 6-week-old plants using an RNasey kit (Qiagen). cDNA was synthesized using the Omniscript Reverse Transcription kit (Qiagen). Primers were designed to amplify the coding regions of the following AAphans: OsAAP16 (Os12g08890), OsAAP7 (Os05g34980), OsAAP3 (Os06g36180) and OsAAP1 (Os07g04180). PCR products were cloned into pCR8/GW/TOPO (Invitrogen) and sequences were confirmed. These clones were recombined with the oocyte expression vector pO02/GW (Sun et al. 2010).

Reverse primers were designed for OsAAP3 and OsAAP1 to amplify coding sequences without stop codons using the pO02 clones as templates. Products were cloned into pCR8/GW/TOPO. The pH-sensitive GFP reporter, pHusion, was amplified by PCR from pMP1922 (Gjetting et al. 2012). A BP recombination reaction was performed to insert the pHusion sequence into pDONR-P2R-P3. The recombination reaction was performed to insert the CaMV 35 S promoter upstream of the OsAAP3 or OsAAP1 coding sequence and the pHusion reporter. The reaction used LR Clonase II Plus from Invitrogen. The resulting vectors were called pB7m34GW-OsAAP3-pHusion and pB7m34GW-OsAAP1-pHusion.

Expression in oocytes

Oocytes were prepared as described previously (Sivitz et al. 2005). Constructs in pO02/GW were linearized using MluI, and cRNA was synthesized using the SP6 mMessage mMACHINE kit (Ambion). Oocytes were injected with 25 ng (50 nl) of cRNA except for the following: for OsAAP1, 60 ng of cRNA was injected per oocyte; for OsAAP3, 2.5 ng of cRNA was injected per oocyte for measurements of lysine and arginine transport and 10 ng was injected per oocyte for all other experiments. Oocytes were incubated in Barth’s solution (88 mM NaCl, 1 mM

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KCl 0.33 mM (NaCl)2, 0.41 mM CaCl2, 0.82 mM MgSO4, 2.4 mM NaHCO3, 10 mM HEPES, pH 7.6) containing 10 μg ml−1 gentamycin for 3–7 d at 15°C prior to recordings.

**Electrophysiology**

Electrophysiology measurements were made using a Dagan TEVC 200A amplifier (Dagan Corp.). Currents were recorded using Clampex (Axon Instruments Inc.). Thin wall 1.5 mm borosilicate glass pipettes (Warner Instruments Corp.) filled with 1 M KCl were used for recordings. Oocytes were perfused with a modified Na Ringer solution (115 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 5 mM MES, pH 5.6 with Tris) and clamped at a holding potential of −40 mV. Solutions of amino acid substrates in the same Na Ringer were prepared and the pH adjusted to 5.6. Substrate-dependent currents were measured at membrane potentials from +57.5 to −137.6 mV in 20 mV increments using 300 ms voltage pulses. Currents were filtered at 2,000 Hz. Background currents measured before and after substrate applications were averaged and subtracted from currents recorded in the presence of substrate. Currents from at least three independent oocytes were recorded for each experiment. The relationship between substrate concentration and substrate-induced current was fitted to the Michaelis–Menten equation:

\[ I = \frac{(I_{max} \times [S])}{K_m + [S]} \]

where \( I \) is the current, \( S \) is the substrate, \( I_{max} \) is the maximal substrate-induced current and \( K_m \) is the substrate concentration when substrate-induced current is half maximal. Non-linear regression was performed using Prism (GraphPad Software).

**Transient expression in onion cells**

Epidermal peels from onion (Allium cepa) were prepared on Murashige and Skoog (MS) plates (0.5 g l−1 MES, 1 g l−1 sucrose, pH 5.7) supplemented with 1% phytagar and 100 μg ml−1 ampicillin. Epidermal peels were bombarded with DNA-coated microparticles using the Bio-Rad Biologic PDS-1000 He as described by Sivitz et al. (2007). After bombardment, epidermal peels were incubated on MS plates wrapped in Parafilm and stored in the dark for 24 h. Confocal microscopy was performed with a Nikon A1-S1 spectral confocal microscope using excitation/emission wavelengths of 488/525 nm for EGFP and 561/595 nm for RFP.

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**Disclosures**

The authors have no conflicts of interest to declare.

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