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Substitution of a single amino acid residue in the aromatic/arginine selectivity filter alters the transport profiles of tonoplast aquaporin homologs ☆

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ABSTRACT

Aquaporins are integral membrane proteins that facilitate the transport of water and some small solutes across cellular membranes. X-ray crystallography of aquaporins indicates that four amino acids constitute an aromatic/arginine (ar/R) pore constriction known as the selectivity filter. On the basis of these four amino acids, tonoplast aquaporins called tonoplast intrinsic proteins (TIPs) are divided into three groups in *Arabidopsis*. Herein, we describe the characterization of two group I TIP1s (TgTIP1;1 and TgTIP1;2) from tulip (*Tulipa gesneriana*). TgTIP1;1 and TgTIP1;2 have a novel isoleucine in loop E (LE2 position) of the ar/R filter; the residue at LE2 is a valine in all group I TIPs from model plants. The homologs showed mercury-sensitive water channel activity in a fast kinetics swelling assay upon heterologous expression in *Pichia pastoris*. Heterologous expression of both homologs promoted the growth of *P. pastoris* on ammonium or urea as sole sources of nitrogen and decreased growth and survival in the presence of H_2O_2. TgTIP1;1- and TgTIP1;2-mediated H_2O_2 conductance was demonstrated further by a fluorescence assay. Substitutions in the ar/R selectivity filter of TgTIP1;1 showed that mutants that mimicked the ar/R constriction of group I TIPs could conduct the same substrates that were transported by wild-type TgTIP1;1. In contrast, mutants that mimicked group II TIPs showed no evidence of urea or H_2O_2 conductance. These results suggest that the amino acid residue at LE2 position is critical for the transport selectivity of the TIP homologs and group I TIPs might have a broader spectrum of substrate selectivity than group II TIPs.

1. Introduction

Aquaporins are integral membrane proteins that are involved in the transportation of water and other low molecular weight substances across biological membranes, and are found in all living organisms [1–2]. Whereas 13 different aquaporins have been identified in mammals [3], a greater diversity of aquaporins has been found in the genomes of plants. For examples, the genomes of rice (*Oryza sativa*), *Arabidopsis thaliana*, maize (*Zea mays*), and the poplar tree (*Populus trichocarpa*) contain 39, 35, 33, and 55 aquaporin homologs, respectively [4–8]. Plant aquaporins are generally classified into four subclasses on the basis of sequence homology and subcellular localization: (i) plasma membrane intrinsic proteins (PIPs), which are localized in the plasma membrane (PM); (ii) tonoplast intrinsic proteins (TIPs), which are localized in the vacuolar membranes (VM); (iii) nodulin-26-like intrinsic proteins; and (iv) small basic intrinsic proteins [9]. However, recently a fifth subclass of uncharacterized intrinsic proteins has been reported [8]. Plant aquaporins are involved in numerous physiological processes, such as cell elongation, adaptation and recovery from water deficit, anoxic stress, transpiration, photosynthesis, water uptake by roots, seed desiccation and germination, maintenance of cell turgor, inhibition of self-pollination, closure of leaf guard cells, and petal movement [9–16]. Functions of the aquaporins have also been associated with plant responses to numerous abiotic stresses, such as drought, osmotic stresses, and other perturbations in environmental conditions [17–18].

Recently, it has been reported that plant aquaporins are transporters not only of water but also of other substrates of physiological significance [2,9,19]. Among the plant aquaporins, TIPs are noted in particular for their ability to transport multiple substrates [20]. In addition to possessing water channel activity (WCA), certain TIPs from rice and tobacco have been reported to transport glycerol [21–22]. Urea conductance has been observed for several TIPs from *A. thaliana* (AtTIPs) [23–25]. The transport of NH_3, which is not substantially

Abbreviations: ar/R, aromatic/arginine; H_2DCFDA, 2,7-dichlorodihydrofluorescein diacetate; NPA, Asn-Pro-Ala; PIP, plasma membrane intrinsic protein; PM, plasma membrane; RACE, rapid amplification of cDNA ends; TIP, tonoplast intrinsic protein; TM, transmembrane; VM, vacuolar membranes; WCA, water channel activity; YNB, yeast nitrogen base

☆ "The nucleotide sequence data reported in this paper and deposited to DDBJ will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB591832 (TgTIP1;1) and AB591833 (TgTIP1;2)".

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larger than water, has been reported for TIP homologs from wheat (TaTIP2;1, TaTIP2;2) [26–27] and *A. thaliana* (AtTIP2;1, AtTIP2;3 and AtTIP1;1) [28–29]. Two TIP homologs from *A. thaliana* (AtTIP1;1 and AtTIP1;2) have been shown to be permeable to H_2O_2 [30], whose size allows it to mimic water. However, although analysis of the genome of *A. thaliana* has revealed 10 putative TIPs [6], the functional activities of AtTIP2;2, AtTIP3;1, and AtTIP3;2 have not been reported to date. Moreover, the molecular mechanisms involved in the conduction of substrates through TIPs remain elusive.

Aquaporins comprise six transmembrane (TM) α-helices (helix H1–H6), which are connected by five loops (loops A–E), with both the N- and C-termini located on the cytoplasmic side of the membrane. They form tetrameric complexes in which each subunit behaves as a functional water channel [9]. The pore of the channel is characterized by two regions of constriction that specify the profile of transport selectivity. The first constriction is formed at the center of the pore by the close opposition of two asparagine residues, which are located in two Asn-Pro-Ala (NPA) motifs on loops B and E [31]. This constriction is involved in proton exclusion [32]. The second constriction is referred to as the aromatic/arginine (ar/R) constriction or the selectivity filter and is formed at the extracellular mouth of the pore by four residues from helix 2 (H2), helix 5 (H5), and loop E (LE1 and LE2), respectively [33–34]. Variability at this site is thought to form the basis of the broad spectrum of substrate conductance that is observed in plant aquaporins [5,8,31,35]. All the PIP homologs from plants that have been studied to date have ar/R selectivity filters that are similar to that of the water-specific mammalian aquaporin, AQP1. In contrast, TIPs show significant diversity within the ar/R region, which suggests that TIPs are more diverse in terms of transport function than PIPs [5,8,31,34]. On the basis of the four key residues in the ar/R selectivity filter, the 10 TIPs found in *A. thaliana* can be classified into three groups: i) group I (all AtTIP1s), ii) group II (all AtTIP2s, AtTIP3s, and AtTIP4;1), and iii) group III (AtTIP5;1) [31]. Group II has two subgroups, namely group IIa, which represents all AtTIP2s, and group IIb, which represents the AtTIP3s and AtTIP4;1. The 17 TIPs found in poplar also fit into these three groups [8]. To explore the mechanism by which NH_3/NH_4^+ and urea are conducted through TIPs, the ar/R site of AtPIP2;1 has been mutagenized to mimic the ar/R selectivity filter in TIPs from *A. thaliana* [29]. All mutants except the TIP2-like mutant conducted urea, but remained impermeable against NH_3/NH_4^+. Given that two native TIP homologs are known to allow ammonia conductance [29], it is clear that the understanding of the factors that dictate selectivity remains insufficient. Therefore, it would be of great value to explore the structure–function relationships of TIPs and the mechanism of selectivity toward different substrates further by mutagenizing the ar/R region of a TIP to mimic the ar/R selectivity filter of different TIP homologs from *A. thaliana* or other model plants.

Previously we have characterized four PIPs from the petals of tulip (*Tulipa gesneriana*) flowers, one of which was proposed to be associated with temperature-dependent and water transport-concomitant petal opening and closing movements [15–16]. TIPs with WCA might play a role in maintaining osmotic equilibrium within vacuoles during the fluctuations in cytosolic volume that may occur during tulip petal movement due to transcellular water transport. In the study reported herein, we have characterized two TIP1 homologs with WCA from tulip petals: TgTIP1;1 (DDBJ/EMBL/GenBank accession no. AB591832) and TgTIP1;2 (DDBJ/EMBL/GenBank accession no. AB591833). In both TgTIP1s, the ar/R selectivity filter had an Ile at the LE2 position, whereas a Val is found at this position in all TIP1 homologs from *A. thaliana*, rice, maize, and poplar [5,8,31]. To investigate this intriguing characteristic of the TgTIP1 homologs, and to mimic the ar/R regions of group I and II TIPs from *A thaliana*, the Ile at LE2 and Ala at LE1 in TgTIP1;1 were mutated and the resulting mutant proteins heterologously expressed in *Pichia pastoris*. The conduction of water, NH_3/NH_4^+, urea, and H_2O_2 by these wild-type and mutant TIP homologs was then studied. The wild-type and mutant TIP homologs showed different transport selectivities and these differences were linked to amino acid substitutions at the LE2 position of the ar/R selectivity filter.

2. Materials and methods

2.1. RNA extraction and cDNA synthesis

Total RNA extracted from the petals of two-day-old tulip (*T. gesneriana*) flowers was used to synthesize first strand cDNA for the isolation of full-length cDNA using methods described previously [16].

2.2. Isolation of gene sequences encoding TIPs

Degenerate primers (forward primer, 5′-TCTACATCATCGCCCAGCTCCTC-3′; reverse primer, 5′-CGTACACAATCCCCGCGATTCCT-3′) were designed using conserved sequences of TIP genes in *A. thaliana*, maize and other plant species. PCR was performed with the cDNA as the template using *Taq* polymerase (TaKaRa Bio, Japan) as described previously [16]. The amplified PCR products were cloned into the pT7Blue T-vector (Novagen, Germany), and then sequenced using an ABI Prism™ 3100-Avant Genetic Analyzer (Applied Biosystems, USA). This sequence information was used to design gene-specific primers (Table 1) for use in the rapid amplification of cDNA ends (RACE) technique to obtain full-length cDNA as described previously [16].

2.3. Construction of TgTIP1;1-G_3-H_6, TgTIP1;2-G_3-H_6, and the mutants of TgTIP1;1-G_3-H_6 and heterologous expression in P. pastoris

Constructs that encoded TgTIP1;1 (TgTIP1;1-G_3-H_6) and TgTIP1;2 (TgTIP1;2-G_3-H_6) with a $(Gly)_3$-$(His)_6$ tag before the stop codon were synthesized using the pPICZ-B expression vector (Invitrogen, USA), transformed into *P. pastoris* strain KM71H (Invitrogen, USA), and heterologously expressed as described previously [36]. Prior to yeast transformation, the TgTIP1;1-G_3-H_6/pPICZ-B and TgTIP1;2-G_3-H_6/pPICZ-B plasmids were linearized with *Pme* I (New England BioLabs Inc., USA). The empty plasmid pPICZ-B, without any expression cassette, was also transformed into the same *P. pastoris* strain. Three single mutants (I201V, I201R, and A195G) and a double mutant (A195G/I201R) of TgTIP1;1-G_3-H_6 were constructed by site-directed mutagenesis as described previously [16] and transformed into the same *P. pastoris* strain. Transformants were selected by plating on YPDS agar (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, and 2% agar) that contained 100 μg/ml zeocin. Integration of the gene of interest into the *P. pastoris* genome was verified by PCR using genomic DNA isolated from the transformants, in accordance with the manufacturer's instructions (Invitrogen, USA). Yeast culture, induction of protein expression, and harvesting of cells were performed in accordance with Daniels and Yeager [36]. The cell pellets were frozen and stored at −80 °C until required.

Table 1
List of primers used for RACE.

Primers	Sequence	Use
3R-TIP1;1	5′- GGCACCGGCACCTTCGGTCTCGT-3′	TIP1;1 3′ RACE
3R-TIP1;2	5′- GGTCTCGTCGCCGGCGTCAGCGT-3′	TIP1;2 3′ RACE
5R-TIP1;1	5′- CTTTCCGCCATCAACCTAGCCAAAAATCAC-3′	TIP1;1 5′ RACE
5R-TIP1;2	5′- GACCGGCATAGTTTGGGGTTGCATTGTG-3′	TIP1;2 5′ RACE
GeneRacer 3′	5′-GCTGTCAACGATACGCTACGTAACG-3′	3′ RACE
GeneRacer 5′	5′-CGACTGGAGCACGAGGACACTGA-3′	5′ RACE