**Solanaceae XIPs are plasma membrane aquaporins that facilitate the transport of many uncharged substrates**

Gerd Patrick Bienert¹, Manuela Désirée Bienert¹, Thomas Paul Jahn², Marc Boutry¹ and François Chaumont¹,*

¹Institut des Sciences de la Vie, Université catholique de Louvain, Croix du Sud 4-15, B-1348 Louvain-la-Neuve, Belgium, and
²Department of Agriculture and Ecology, Faculty of Life Sciences, University of Copenhagen, Thorvaldensvej 40, 1871 Frederiksberg, Denmark

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*For correspondence (fax +32 10 473872; e-mail francois.chaumont@uclouvain.be).

**SUMMARY**

Major intrinsic proteins (MIPs) transport water and uncharged solutes across membranes in all kingdoms of life. Recently, an uncharacterized MIP subfamily was identified in the genomes of plants and fungi and named X Intrinsic Proteins (XIPs). Here, we describe the genetic features, localization, expression, and functions of a group of Solanaceae XIPs. XIP cDNA and gDNA were cloned from tobacco, potato, tomato, and morning glory. A conserved sequence motif in the first intron of Solanaceae XIPs initiates an RNA-processing mechanism that results in two splice variants (α and β). When transiently or stably expressed in tobacco plants, yellow fluorescent protein-tagged NtXIP1;1α and NtXIP1;1β were both localized in the plasma membrane. Transgenic tobacco lines expressing NtXIP1;1-promoter-GUS constructs and RT-PCR studies showed that NtXIP1;1 was expressed in all organs. The NtXIP1;1 promoter was mainly active in cell layers facing the environment in all above-ground tissues. Heterologous expression of Solanaceae XIPs in Xenopus laevis oocytes and various Saccharomyces cerevisiae mutants demonstrated that these isoforms facilitate the transport of bulky solutes, such as glycerol, urea, and boric acid. In contrast, permeability for water was undetectable. These data suggest that XIPs function in the transport of uncharged solutes across the cell plasma membrane in specific plant tissues, including at the interface between the environment and external cell layers.

**Keywords:** aquaporin, X intrinsic protein, transport, substrate selectivity, solute, alternative splicing.

**INTRODUCTION**

Proteins of the Major Intrinsic Protein (MIP) superfamily are channels forming a hydrophilic pathway for small uncharged molecules across the lipid bilayer of biological membranes (Gomes et al., 2009). Based on both, sequence similarities and major transport activities, MIPs can be subdivided into two clades, the water-permeable aquaporins (AQPs) and the glycerol-conducting aquaglyceroporins (Heymann and Engel, 1999). Despite the sometimes large sequence difference between MIPs from different clades, the overall structure is highly conserved. MIPs form tetramers, in which each monomer is composed of six transmembrane helices (TMHs) connected by five loops (A–E) and two membrane-embedded half-helices, each containing the highly conserved MIP asparagine-proline-alanine (NPA) signature sequence. These motifs meet in the middle of the membrane, forming a narrow hydrophilic path (Murata et al., 2000). A second narrow selectivity filter, the so-called aromatic/arginine (ar/R) constriction region, is formed by four amino acids (residues R1–4) that contribute to a size exclusion barrier and a hydrogen bond environment necessary for the effective transport of a substrate (Murata et al., 2000). The amino acids forming this tetrad are found in TMH 2 (R1), TMH 5 (R2), and loop E (R3 and R4).

One remarkable difference between MIPs from plants and other organisms is the much larger isoform diversity in plants. While 13 isoforms have been identified in mammals (Agre and Kozono, 2003), many more have been found in the genomes of higher plants, with, for instance, 35 in Arabidopsis (Johanson et al., 2001), 55 in poplar (Gupta and Sankaramakrishnan, 2009), 71 in cotton (Park et al., 2010) and at least 36 in maize (Chaumont et al., 2001). Even the genome of the evolutionarily early land plant Physcomitrella patens encodes 23 different isoforms (Danielio and Johanson, 2008). Plant MIPs play key functions in transmembrane water conductance in a wide variety of physiological processes, such as uptake from the soil, transcellular...
and root-to-shoot transport, cellular water homeostasis and osmotic driven growth control (for reviews, see Hachez and Chaumont, 2010; Heinen et al., 2008; Maurel et al., 2008; Tyerman et al., 2002). However, recent studies have revealed that some isoforms play important roles in many other processes, such as gas and nutrient uptake and translocation, metalloid homeostasis, nitrogen remobilization and signal transduction (Kaldenhoff and Fischer, 2006; Bienert et al., 2008a; Gomes et al., 2009; Bienert and Chaumont, 2010; Soto et al., 2010).

MIPs of higher plants belong to the AQP clade and are subdivided into five evolutionarily distinct subfamilies, the plasma membrane intrinsic proteins (PIPs), the tonoplast intrinsic proteins (TIPs), the small basic intrinsic proteins (SIPs), the nodulin26-like intrinsic proteins (NIPs), and the uncharacterized X intrinsic proteins (XIPs) (Chaumont et al., 2001; Johanson et al., 2001; Danielson and Johanson, 2008). The XIP subfamily was recently identified in the genomes and/or expressed sequence tag (EST) libraries of different non-vascular and vascular plants, including P. patens, poplar, tomato, cotton and grapevine (Danielson and Johanson, 2008; Gupta and Sankararamakrishnan, 2009; Sade et al., 2009; Shelden et al., 2009; Park et al., 2010). Interestingly, XIPs are present in a wide variety of eudicot plant species, but not in Brassicaceae (e.g. Arabidopsis), and are so far not detected in monocots (Danielson and Johanson, 2008). XIP genes have also been identified in the genomes of many fungi and a protozoan species (Gupta and Sankararamakrishnan, 2009). The plant, moss, protozoa, and fungi XIPs form a clear monophyletic group distinct from the other MIP subfamilies, suggesting specific roles in solute transport.

The most highly conserved feature of XIP protein sequences, which could be used as a signature for this subfamily, is the NPARC motif, with a cysteine residue located after the second NPA motif (Figure 1) (Danielson and Johanson, 2008; Gupta and Sankararamakrishnan, 2009). In addition, XIPs from plants and fungi show considerable amino acid variation at both the first NPA motif and the ar/R filter. This variability is also observed in NIPs and suggests that XIPs probably became diversified in terms of substrate specificity, while conserving other MIP-specific sequence features. Based on the four amino acids defining the ar/R filters, XIPs from eudicots can be divided into four subclasses.
(Gupta and Sankararamakrishnan, 2009), two of which have an ar/R signature similar to that found in some plant NIPs, while the other two are even more hydrophobic. To elucidate the function of plant XIPs, we undertook a comprehensive molecular and functional characterization of XIPs from tobacco (Nicotiana tabacum), potato (Solanum tuberosum), and tomato (Solanum lycopersicum). Cloning of XIP cDNAs and genomic DNAs allowed us to identify an mRNA splicing mechanism that results in two XIP protein variants per species. The proteins were localized in the plasma membrane and, when heterologously expressed in Xenopus oocytes or yeast, facilitated the diffusion of a number of small solutes, but not water. Furthermore, the expression pattern of NtXIP1;1 in tobacco plants was analyzed.

RESULTS

Cloning of XIP cDNAs from Nicotiana tabacum and Solanum tuberosum

ESTs encoding parts of the XIP proteins from Nicotiana benthamiana, Populus trichocarpa and Vitis vinifera, identified by Danielson and Johanson (2008), were used as query sequences in a blast search of publicly-available EST databases for Solanales species. Several ESTs encoding putative XIP proteins were identified (Table S1) and used to design primers to clone the full-length cDNAs. The usage of one primer pair resulted in the amplification of two different cDNAs from N. tabacum and S. tuberosum, designated as XIP1;1a and XIP1;1b, respectively. NtXIP1;1a and StXIP1;1a encode a 325 amino acid protein, while NtXIP1;1b and StXIP1;1b encode a 326 amino acid protein. The a- and b-isoforms of the respective species are identical, except that asparagine 31 and arginine 32 of NtXIP1;1b and StXIP1;1b are replaced by a lysine residue in NtXIP1;1a and StXIP1;1a (Figure 1).

Cloning of genomic XIP sequences from Solanales species

To gain further insights into the gene structure of tobacco and potato XIPs, we cloned the respective genomic counterparts. This resulted in the identification of a single genomic XIP fragment in both plant species. These fragments have the same intron–exon organization (Figure 2a,b). Two introns are present at identical positions in the two species in the region encoding the cytoplasmic N-terminus. The first intron is inserted into a codon, while the second is located between two codons, in frame with the coding sequence. Like nearly all eukaryotic nuclear introns, they have the canonical dinucleotides GT and AG in their donor and acceptor splice sites. A comparison between the genomic sequence and those of the two cDNAs suggests that the two protein variants result from an alternative splicing event (Figure 2). NtXIP1;1a and StXIP1;1a can be transformed into NtXIP1;1b and StXIP1;1b, respectively, simply by shifting the first intron acceptor

Figure 2. Gene structure and alternative splicing sites of Solanales XIP1;1s. (a–d) Genomic structure of NtXIP1;1s (Nt; Nicotiana tabacum) (a); StXIP1;1s (St; Solanum tuberosum) (b); SlXIP1;1s (Sl; Solanum lycopersicum) (c); or InXIP1;1s (In; Ipomoea nil) (d). Horizontal gray bars represent exons and the gaps introns. The lengths, in nucleotides, of the different exons and introns are indicated. The first exon-intron-exon borders are shown enlarged and the nucleotide and protein sequences are displayed. The sequences of the a and b forms for each species are directly juxtaposed to indicate the shift in the splice site resulting in the two splice variants. The canonical dinucleotide intron borders (GT and AG) are underlined. (e) Schematic representation of the phylogenetic conservation of the alternative splicing in Solanaceae XIP1;1 genes.
splice site three nucleotides upstream. This truncation of the intron without disturbing the open reading frame is possible because of the presence of two AG dinucleotides separated by one nucleotide (Figure 2).

To determine whether this alternative splicing mechanism is restricted to certain plant families, such as the Solanaceae, or whether it is conserved across species, the genomic sequences of S. lycopersicum (tomato) and Ipomoea nil (morning glory) were cloned and sequenced (Figure 2c,d). Tobacco, potato, tomato, and morning glory belong to the same Solanales order. While tomato, like tobacco and potato, is a member of the Solanaceae family, morning glory belongs to the family of the Convolvulaceae (Figure 2e). As in the tobacco and potato XIP genes, the tomato XIP exhibits an AG dinucleotide repeat at the boundary between the first intron and second exon (Figure 2). The cloning and sequencing of the tomato XIP cDNAs confirmed the existence of both splice variants (SlXIP1;1a and SlXIP1;1b). SlXIP1;1a is identical to tomato mRNA GenBank identifier BT014197. In contrast, the end of the first intron in the two P. patens XIP genes, the six P. trichocarpa XIP genes, and the newly cloned I. nil XIP gene does not possess a repeated AG dinucleotide motif, preventing alternative splicing (Figures 2d,e and data not shown). Consistent with this finding, only a single cDNA (InXIP1;1) was amplified from I. nil RNA.

Analysis of the XIP protein sequences

A phylogenetic tree including the XIPs and representative members of the four other known dicot MIP subfamilies (PIPs, TIPs, NIPs, and SIPs) from P. trichocarpa and N. tabacum was obtained (Figure 3). All cloned Solanales XIPs cluster together with PtXIPs and VvXIPs, and are grouped separately from the PIP, TIP, NIP, and SIP subfamilies.

All XIP isoforms have characteristic sequence motifs found throughout the MIP family. The two highly conserved NPA signature motifs are found, but the alanine in the first NPA motif is replaced by valine or isoleucine in the Solanales isoforms (Figure 1).

The four residues forming the ar/R selectivity filter of the Solanales XIP1s are isoleucine from TMH 2, threonine from TMH 5, and alanine and arginine from loop E (Figure 1). This pore layout is similar to that of the ar/R filter of the phylogenetic NIP3 group, which is made up of a threonine/serine/alanine from TMH 2, valine/iseoleucine from TMH 5, and glycine/alanine and arginine from loop E, except that the residues from TMH 2 and TMH 5 are interchanged and differ slightly (Figure 1).

Loop C of the Solanales XIPs contains a LGGC motif that is highly conserved in plant XIPs (Danielson and Johanson, 2008). The other most conserved feature of XIPs, the NPARC motif with a cysteine residue located after the second NPA motif, is also present (Figure 1). The N-termini of XIPs differ substantially from those of other MIP subfamilies.

NtXIP1;1 and StXIP1;1 are localized in the plasma membrane

To investigate the subcellular localization of NtXIP1;1 in plant cells and to determine whether the different splice variants are trafficked to different subcellular membranes, YFP–NtXIP1;1a and YFP–NtXIP1;1b were transiently or stably expressed in tobacco. Both fusion proteins were detected as a thin ring at the periphery of epidermal cells and were co-localized with the fluorescence of the dye FM4-64, a
marker labeling the plasma membrane shortly after its infiltration (Figure 4a–f). Similar data were obtained with the potato YFP–StXIP1;1α (Figure S1). Plasma membrane localization of YFP–NtXIP1;1α was also observed in cells of the root apical meristem from transgenic tobacco (Figure 4g). Together, these results indicate that tobacco and potato XIPs localize in the plasma membrane and that the alternative splicing has no effect on the subcellular localization under the test conditions.

**Expression of NtXIP1;1 in organs and tissues**

A 5’ 1100-bp fragment of the NtXIP1;1 gene, containing 107 bp of the NtXIP1;1 coding sequence and 993 bp of the ATG-upstream sequence, was amplified by inverted PCR from genomic DNA. A stop codon in frame at –39, a TATA-box variant (TATATT) at –58, and two putative TATA-boxes (TATATAA) at –292 and –344 were identified (data not shown).

Tobacco plants expressing the ‘β-glucuronidase GUS’ – ‘fluorescent Venus protein’ fusion gene under the control of the NtXIP1;1 promoter were used to identify the cell type in which NtXIP1;1 is expressed. GUS activity was observed in the tips of the primary and lateral roots, but not in the root elongation zone (Figure 5h–j). However, a GUS signal was seen in the mature part of the root (Figure 5h). In cross-sections of this zone, GUS activity was observed in the cortex, but not in the vascular tissues (Figure 5i). In the stem, GUS activity was detected in the epidermal and adjacent subepidermal cell layers, but not in the vascular tissues and surrounding cortex cells (Figure 5c). In the leaf, GUS staining was detected in guard cells and in the epidermal and adjacent subepidermal cell layers (Figure 5d–f). NtXIP1;1 promoter activity in epidermal cells was verified using a confocal microscope to detect Venus fluorescence, which was observed in the cytoplasm and the nucleus (Figure 5g). No GUS activity was detected in vascular tissues or in further inner cell layers (Figure 5c–f,h,i). In the flowers, a GUS signal was detected in the upper part of the petal and the surface of the peduncle (Figure 5a,b). Expression of NtXIP1;1 in the organs of wild-type N. tabacum plants was confirmed using RT-PCR. NtXIP1;1 was expressed in all organs, including the root, stem, leaf, and flower, regions in which promoter GUS activity was also detected (Figure 5k).

**Tobacco and potato XIPs do not channel water in Xenopus oocytes**

The water channel activity of NtXIP1;1α and β and StXIP1;1α alone or fused to YFP was tested by heterologous expression in Xenopus oocytes. High osmotic water permeability coefficient (P) values were obtained for cells expressing the positive control ZmPIP2;5 (Chaumont et al., 2000). In contrast, no significant P increase was seen for cells expressing the different XIPs (Figure 6a). To exclude the possibility that the lack of a swelling response was due to failure to express the protein or to disturbed trafficking to the plasma membrane, the localization of the fluorescent-tagged isoforms was investigated (Figure 6b). Cells expressing the different XIP constructs showed a sharp YFP fluorescence at their periphery, similar to cells expressing YFP–ZmPIP2;5, which is localized to the plasma membrane (Fetter et al., 2004). These data indicate that NtXIP1;1 and StXIP1;1 do not facilitate water transport (Figure 6a,b).

**Tobacco XIPs facilitate the transport of glycerol in Xenopus oocytes**

The ar/R selectivity filter of the Solanaceae XIP1;1s resembles the filter of NIP3 isoforms that have been shown to transport uncharged molecules of bigger volume than water, such as glycerol, urea, and metalloids (Wallace and Roberts, 2004; Bienert et al., 2008a). To examine the glycerol permeability of NtXIP1;1, we measured [14C] glycerol accumulation in oocytes expressing NtXIP1;1α, NtXIP1;1β, or rAQP9 as a positive control (Tsukaguchi et al., 1998) (Figure 6c). These oocytes accumulated 6- to 12-fold more...
glycerol than water-injected control oocytes when incubated for 10 min with 1 mM glycerol, indicating that NtXIP1;1 is an efficient glycerol transporter. The positive transport result with glycerol supports that the protein is correctly folded and localized to the plasma membrane, and hence strengthens the negative result obtained with the water transport assay.

**XIPs facilitate the transport of urea in Saccharomyces cerevisiae and Xenopus oocytes**

To determine whether XIPs also facilitate the diffusion of urea across membranes, a yeast complementation assay was performed. The yeast mutant YNVW1, which carries a deletion in the DUR3 urea transporter gene and is unable to grow on medium containing 5 mM or lower concentrations of urea as the sole nitrogen source (Liu et al., 2003), was transformed with an empty vector or a vector containing NtXIP1;1α, NtXIP1;1β, StXIP1;1α, StXIP1;1β, rAQP9, or At-DUR3. All yeast transformants were able to grow on 1 mM arginine, as arginine uptake is not impaired in the YNVW1 yeast mutant (Figure 7a). Passive diffusion of urea across the lipid bilayer at the concentration of 20 mM urea was sufficient to feed all yeast transformants. On medium containing 2 or 4 mM urea, the complementation phenotype was seen for yeast expressing AtDUR3, rAQP9, or the Solanaceae XIPs. Yeast cells expressing rAQP9 were not able to grow on medium containing 1 mM urea, but cells expressing a XIP from tobacco or potato grew at this concentration, strongly suggesting that XIPs transport urea across yeast membranes.

To verify this transport activity of XIPs, an uptake assay using [14C]-labeled urea was performed in the oocyte system (Figure 7b). Urea uptake after 10 min of incubation with 1 mM urea was 8- to 13-fold higher in oocytes expressing NtXIP1;1α or β than in control oocytes, confirming that XIPs facilitate urea transport (Figure 7b).

**XIPs increase the sensitivity of yeast cells to hydrogen peroxide**

It has been shown that the essential signaling molecule H2O2 crosses biological membranes via AQPs (Bienert et al., 2007; Dynowski et al., 2008b). To test whether XIPs can mediate the transport of H2O2, the D_dur3 and D_mep1-3 yeast mutant strains were transformed with vectors expressing the different NtXIP1;1, SIXIP1;1 or StXIP1;1 isofoms or hAQP8.
as a positive control (Bienert et al., 2007) (Figure 8). In yeast containing the empty vector, addition of 1 mM H₂O₂ did not decrease cell growth. However, expression of tobacco, tomato or potato XIPs markedly reduced cell survival on medium containing H₂O₂ to a similar extent as that of the positive control hAQP8. Expression of AtDUR3, rAQP9, or TaTIP2;1 did not decrease, or only slightly decreased, yeast growth on medium containing H₂O₂, demonstrating that the increased sensitivity of yeasts expressing XIPs was not due to modifications of the membrane permeability as the result of the expression of a membrane protein per se.
XIPs increase the sensitivity of yeast cells to externally supplied boric acid

To determine whether XIPs can channel boric acid, a yeast mutant (Δfps1) with a deletion of one of its endogenous aquaglyceroporins, ScFps1p, was used (Nozawa et al., 2006; Fitzpatrick and Reid, 2009). Expression of the different splice variants of Solanaceae XIPs in the Δfps1 strain impaired growth on medium containing 10 mM boric acid and completely inhibited growth in the presence of 20 mM boric acid (Figure 9). In contrast, no effect on the growth of yeast transformed with an empty vector was seen on plates containing 20 mM of boric acid, whereas total growth inhibition was observed at 50 mM boric acid. This increase in sensitivity to boric acid indicates that XIPs can facilitate the transport of this metalloid. AtNIP5;1 a plant boron channel protein (Takano et al., 2006), did not render yeast more sensitive to boron (Figure 9).

DISCUSSION

In this study, we isolated and functionally characterized members of the plant XIP subfamily of MIPs. The XIP1;1 gene was isolated from tobacco, potato, tomato and morning glory. All the identified Solanales XIP proteins clustered with other known XIP sequences, which are phylogenetically separated from the other plant MIP subfamilies.

Splicing variants

Two XIP1;1 cDNAs (α and β), differing in length by only one codon, were identified in N. tabacum, S. tuberosum, and S. lycopersicum. Comparison of the cDNA and genomic sequences suggested that the codon addition in XIP1;1β was due to alternative splicing of the mRNA at the first intron-exon junction. Interestingly, this RNA processing led to a minimum change in the resulting proteins (Figure 1).

In general, prevalent and non-prevalent splice variants can be observed in constitutively spliced genes. They are
often associated with different subcellular localizations or affect protein function, transport activity, posttranslational modifications, or protein stability (Sheth et al., 2006; Reddy, 2007; Schuler, 2008; Labadorf et al., 2010). As discussed in more detail below, NtXIP1;1x and NtXIP1;1z did not differ in terms of subcellular localization or substrate selectivity. Further studies are required to quantify precisely the levels of both transcripts in different organs and tissues or in response to different environmental growth conditions, and to elucidate their physiological significance.

Subcellular localization and tissue-dependent expression of NtXIP1;1

The fluorescence distribution of YFP–NtXIP1;1x and -1z transiently and stably expressed in tobacco demonstrated plasma membrane localization for both proteins. XIP EST data from Populus and Nicotiana species, do not indicate a specific expression pattern for XIP genes (Danielson and Johanson, 2008; Gupta and Sankaramakrishnan, 2009). In agreement with this, we succeeded in amplifying NtXIP1;1 cDNAs from various tobacco organs. Interestingly, the NtXIP1;1 promoter seems to be active in all the above-ground tissues facing the environment. This localization might point to a role in solute exchange with the extracellular space or the environment. Furthermore, the absence of promoter activity from vascular tissues is in accordance with the missing expression of all PtXIPs in the differentiating xylem (Gupta and Sankaramakrishnan, 2009), suggesting that NtXIP1;1 is not involved in the loading or unloading of solutes to or from the vascular tissues.

Ar/R selectivity filter and substrate specificity

The four residues forming the ar/R constriction region are crucial for MIP substrate selectivity. When aligning the Solanales XIPs with other plant AQPs, the selectivity filter is made up of isoleucine (R1), threonine or valine (R2), alanine (R3), and arginine (R4) (Figure 1). With an aliphatic amino acid residue in position R1, a small residue in R2, and an alanine in R3, these residues form a wide, rather hydrophobic pore environment that resembles the ar/R region of the NIP3 group, which has been shown to facilitate the diffusion of urea, glycerol, and metalloids but is rather weakly permeable to water (Wallace and Roberts, 2004; Takano et al., 2006; Bienert et al., 2008a; Dynowski et al., 2008a). These structural features suggest that Solanales XIPs are not predominantly water channels and that the transported solute is more bulky and hydrophobic.

Glycerol, urea and ammonia

Consistent with this prediction, our functional analyses clearly demonstrated that Solanales XIPs promote the transport of solutes in a comparable way to NIPs. In oocytes, Solanaeae XIP1;1s showed no water permeability, but conducted radio-labeled glycerol and urea. The transport of urea was confirmed in the yeast complementation assay. Glycerol is a representative substrate for all the non-water conducting MIPs and also represents a common substrate for NIPs. Although several isoforms of diverse MIP sub-families are able to conduct glycerol, there is no physiological evidence for a MIP-mediated glycerol transport in plants. Whether XIPs play this role needs to be investigated directly in plants. The physiological function of XIP-mediated urea permeability in planta remains also to be shown.

The ammonia molecule is slightly larger and more hydrophobic than water. Although these chemical features accommodate, to some extent, the requirements to permeate through a XIP pore, a marginal promotion of growth on medium containing ammonium as sole nitrogen source showed that the tested XIPs minimally conduct ammonia (Figure S2). This supports our notion that Solanaceae XIPs are only weakly permeable for small polar molecules due to the make up of their pore. XIPs probably do not function as high capacity ammonia channels in plants.

Hydrogen peroxide

Expression of Solanaceae XIPs increased the sensitivity of yeast to externally supplied H2O2. Growth inhibition was monitored at similar concentration levels to those that inhibit the growth of yeast expressing hAQP8, which is known to be H2O2-permeable (Bienert et al., 2007). AQPs such as TaTIP2;1 and rAQP9 are less permeable to H2O2 and, consequently, their expression did not result in a similar decrease in growth to that seen with hAQP8 or the Solanaceae XIPs. This clearly indicates that XIP-mediated H2O2 influx caused the growth inhibition phenotype. This H2O2 permeability is surprising, as the same XIPs failed to conduct water to any significant extent. A comparison of the structural and electrostatic characteristics of water and H2O2 revealed their high similarity, especially with respect to properties determining solute permeability through MIPs (Bienert et al., 2006). Until now, all studies dealing with MIP-mediated H2O2 transport have shown that this transport goes along with water permeability. Our substrate specificity data for XIPs provide the first example of MIPs with a high H2O2 permeability, but negligible water permeability. The make up of the ar/R selectivity filter alone definitely does not explain this observation. Testing the water and H2O2 transport activity of XIPs mutated in specific amino-acid residues should give some clues about this intriguing feature.

The epidermis and subepidermal cell layers, which showed high XIP expression, are tissues in which H2O2 is typically generated in response to several biotic and abiotic factors, such as light stress, temperature stress, wounding, or pathogen attacks (Neill et al., 2002). In these responses, H2O2 plays multiple functions as a messenger processing the perceived stimuli and inducing responses in the plant via differential gene expression (Vandenbroucke et al., 2008), direct regulation of protein functions (Møller et al.,
2007), or alterations in cell wall modification processes (Liszkay et al., 2004), or by acting as a defense compound (Torres, 2010). In all these processes, H₂O₂ levels have to be highly controlled in order to induce the physiological response with no detrimental side effects. A channel that facilitates movement of H₂O₂ across the plasma membrane with no detrimental side effects. A channel that can be highly controlled in order to induce the physiological role of XIPs in plants in order to conclude whether XIPs and NIPs share common functions in vascular plants. The overlapping substrate spectrum and the similar pore layout do not necessarily imply a redundant function, but are definitely striking.

**Boric acid**

Expression of either splice variant of NtXIP1;1 or StXIP1;1 greatly increased the sensitivity of Δfps1 yeast mutants to externally supplied boric acid. So far, no positive control for this assay is available. All known active boron transporters are efflux transporters, and the boric acid-permeable MIP, AtNIP5;1, which conducts boron in Xenopus oocytes and in planta (Takano et al., 2006), failed to cause boron sensitivity of yeast, although we used AtNIP5;1Δ47, which has been shown to be functionally expressed in this system (Bienert et al., 2008b). This missing AtNIP5;1 phenotype was also observed in another study (Dynowski et al., 2008a). To exclude a toxic pH effect of the boric acid added to the medium on the growth of yeast expressing XIPs, the survival assay was carried out on both buffered (pH 5.5) and non-buffered medium (data not shown) with consistent results. This strengthens the interpretation that boric acid itself decreases the growth of the yeast and that XIP isomers are permeable to boric acid. The selectivity filter of the physiologically important boron transporters AtNIP5;1 and AtNIP6;1 has alanine at R1, isoleucine at R2, glycine/alanine at R3, and arginine at R4 and chemically resembles those of the XIPs. The expression of NtXIP1;1 in the epidermis and subepidermal tissues of tobacco stems and leaves is very interesting with regard to a potential boron transport capacity. The epidermal cell walls largely contribute to the mechanical properties of these tissues. Homogalacturonans and rhamogalacturonans are major components of these cell walls, and it has been shown that boron crosses these polysaccharides, a physiological process important for cell wall integrity (Miwai and Fujiwara, 2010). Translocation of boron via XIP proteins to cells forming secondary cell walls could therefore be advantageous.

**Do XIP proteins have a similar function to NIPs?**

The sequence and functional data for the Solanaceae XIPs indicate that they share similarities with members of the NIP subfamily, and, more particularly, with the NIP3 group. Proteins from both subfamilies are plasma membrane-localized, have an alanine substitution in the NPA motifs, and have amino acids with similar chemical properties in the α/R selectivity filter. In accordance with a similar selectivity filter composition, the identified substrates channeling through XIPs, such as glycerol or the metalloid boron, are also typical substrates for NIPs, while both XIPs and NIPs are not highly permeable to water. The reason why XIPs seem not to be present in Arabidopsis and the monocots studied so far could be due to a functional redundancy that occurred during evolution. NIPs and XIPs are clearly phylogenetically separated groups. Over time, convergent evolution in terms of channel specificity could have occurred for XIPs and NIPs in vascular plants. In plant species in which a specific role has been acquired, XIPs have been conserved, while, in monocots and Arabidopsis, due to the functional redundancy with NIPs, they have been lost. It will be interesting to determine the physiological role of XIPs in plants in order to conclude whether XIPs and NIPs share common functions in vascular plants. The overlapping substrate spectrum and the similar pore layout do not necessarily imply a redundant function, but are definitely striking.

**EXPERIMENTAL PROCEDURES**

**Cloning of XIP cDNA and gDNA and plasmid constructions**

The XIP encoding query sequences CK295158 (N. benthamiana), 557139, 829126, 767334 759781, 821124 (P. trichocarpa), BT014197 (S. lycopersicum), and AM455454 (V. vinifera) (Danielson and Johanson, 2008), were used to screen EST databases for diverse Solanaceae species (tobacco, potato, tomato, morning glory) (http://compbio.dfci.harvard.edu/tgi/plant.html). Primers (Table S2) matching the identified EST sequences were used to PCR amplify cDNA prepared from 3-week-old tobacco (N. tabacum cv. Petit Havana SR1), morning glory, or tomato seedlings or mature potato plants or gDNA isolated from the four plant species. The PCR products were directionally sub-cloned using a uracil excision-based improved high-throughput USER cloning technique (Nour-Eldin et al., 2011) into the USER-compatible Xenopus expression vectors pNB1u and pNB1YFPu (N-terminal fusion of YFP to the protein of interest) (Nour-Eldin et al., 2006) or the yeast expression vector pYeDP60u (Hamann and Moller, 2007). Seven full-length cDNA sequences were obtained and submitted to GenBank: NtXIP1;1 (HM475295), NtXIP1;1β (HM475294), StXIP1;1α (HM475297), StXIP1;1β (HM475298), SIXIP1;1α (HM475300), SIXIP1;1β (HM475299), and InXIP1;1 (HM475296). Furthermore, four genomic sequences, one for each plant species, were amplified and verified by DNA sequencing.

To clone the promoter region of NtXIP1;1, 10 µg of tobacco gDNA was digested with HindIII or Scal and purified. The linearized DNA was self-ligated using T4 DNA ligase at 16°C for 16 h and purified. iPCR reactions were performed on 100 ng of circularized DNA using HiFi polymerase (Roche) and specific primers (Table S2). A 1100-bp fragment containing 993 bp of the NtXIP1;1 promoter sequence was obtained. The NtXIP1;1 promoter was inserted using NotI and HindIII in front of the GUS-Venus coding sequence in pAUX3131 (Navarre et al., submitted). The fusion construct was then excised using I-Scel and inserted into the pZPP-RC52-tntf plant expression vector (Goderis et al., 2002). The constructs were then used for tobacco (N. tabacum cv. Petit Havana SR1) leaf disc transformation.

pCAMBIA2300 35S (Nour-Eldin et al., 2006) was used to fuse XIP cDNAs to the sequence encoding monomeric YFP to generate pCAMBIA2300 35S N-term mYFP–XIP constructs. The cDNAs of Solanaceae XIPs were amplified by PCR from their respective pNB1u plasmids, using specific primers (Table S2). The constructs were then used for tobacco leaf disc transformation or transient expression in tobacco leaf.
Subcellular localization and imaging

Transient expression of mYFP-XIP proteins in tobacco leaves was performed as described previously (Batoko et al., 2000). Confocal images of transfected tobacco or oocyte cells were acquired 2-3 days after leaf infiltration or oocyte injection using a Zeiss 710 confocal microscope (Carl Zeiss, Jena, Germany). The YFP was excited with the 514 nm line of an argon multimaser and the emitted YFP fluorescence detected between 530 and 570 nm. FM4-64 was excited with the 514-nm line of an argon multimaser.

GUS staining

Various tissues of tobacco plants were fixed and stained as described in Moria et al. (1999). The chemical reaction was carried out at 37°C for at least 1 h, depending on the tissue.

In vitro RNA synthesis and oocyte transport assays

Ready-to-use capped complementary RNAs encoding N-terminal YFP-tagged or non-tagged XIPs were synthesized in vitro as described previously (Fetter et al., 2004). Xenopus laevis oocytes were isolated, defolliculated, and injected, and the osmotic water permeability coefficient (P) determined as described previously (Fetter et al., 2004). Glycerol uptake assays were performed as described previously (Gustavsson et al., 2005). Urea uptake was measured in the same way using [14C]urea (57 μCi mol⁻¹; Amersham Pharmacia Biotech).

Yeast strains and growth assays

For the urea complementation assay, the dur3 mutant YNWV1 S. cerevisiae strain (Liu et al., 2003) was transformed with the vectors described above. In addition, YNWV1 was transformed with empty p426HXT7 vector or p426HXT7 carrying AtDUR3. Transformants were selected on synthetic medium containing 2% agar, 2% glucose, pH 5.5, and yeast nitrogen base (YNB) without amino acids and ammonium (Difco), supplemented with 1 mM arginine. Transformants were spotted on synthetic galactose medium supplemented with 1 mM arginine or different concentrations of urea as sole nitrogen source.

For the boric acid growth assay, S. cerevisiae strain $\mu$ps1 (Fitzpatrick and Reid, 2003) was transformed with the vectors described above. Transformants were selected on synthetic medium containing 2% agar, 2% glucose, pH 5.5, and yeast nitrogen base (YNB) without amino acids and ammonium (Difco), supplemented with 1 mM arginine. Transformants were spotted on synthetic galactose-medium supplemented with 1 mM arginine or different concentrations of boric acid.

For the ammonia complementation assay, S. cerevisiae strain 31019b (Marini et al., 1997) was transformed with either pYeDP60u or pYeDP60u carrying cDNAs for different MIPs. Growth of the transformants was tested as described by Jahn et al. (2004).

For H2O2 growth assays, all the above-described yeast strains were grown with their respective synthetic media containing amino acids according to the autotrophic requirements and supplemented with different concentrations of H2O2.

After 5-11 days of incubation at 28°C, differences in growth and survival in the different assays were recorded. All yeast growth assays were repeated in at least three independent experiments with consistent results.

Phylogenetic analysis

The program CLUSTALW (Thompson et al., 1994) was used to perform multiple sequence alignments of MIP proteins, which were then used to compute a distance matrix using Phylip prodist and construct the evolutionary tree. The stability of branches in the resulting trees was tested by 1000 bootstrap trials. Used sequences can be found in the GenBank data libraries or the Joint-Genome-Institute -JGI- [genome.jgi-psf.org] browser under accession numbers: NtXIP1;1α, HM475295; NtXIP1;1β, HM475294; StXIP1;1α, HM475297; StXIP1;1β, HM475298; StXIP1;1γ, HM475300; StXIP1;1δ, HM475298; InXIP1;1, HM475296; GhXIP1;1, GU989884; rAOQP9, NM_022960.2; hAQ81, NM_001169.2; AtDur3, AT5G45380; AtNIP6;1, AT4G10380; TaTIP2;1, AY526583; NtTIP1, BA959671.1; NtTIPa, CAH24073; NtTIP1;1, AAL33585.1; NtAQP1, AA881801.1; NtNIP2;1, AAL33586.1; PtNIP1;1, 7245206(GI); PtNIP1;2, 6556216(GI); PtNIP2;2, 821084(GI); PtNIP2;2, 648808(GI); PtNIP2;3, 577637(GI); PtNIP2;3, 759787(GI); PtTIP3;3, 708017(GI); PtTIP2;1, 729942(GI); PtTIP2;2, 734665(GI); PtTIP3;1, 584517(GI); PtTIP2;1, 548890(GI); PtTIP4;1, 5617590(GI); PtTIP1;1, 5492121(GI); PtXIP1;1, 557138(GI); PtXIP1;5, 821124(GI); PtXIP1;1, 829126(GI); PtXIP2;1, 759781(GI); PtXIP1;3, 759781(GI); PtXIP4;1, 767334(GI).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Plasma membrane localization of StXIP1;1a fused to YFP in tobacco cells.

**Figure S2.** Ammonia permeability in yeast cells expressing Solanaceae XIP proteins identified by a homology search.

**Table S1.** ESTs encoding putative Solanaceae XIP proteins identified by a homology search.

**Table S2.** Primers used in this study.

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bi-directional diffusion of As(OH)$_3$ and Sb(OH)$_3$ across membranes. BMC Biol. 6, 26.


