RESEARCH PAPER

Insights into *Populus* XIP aquaporins: evolutionary expansion, protein functionality, and environmental regulation

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Abstract

A novel category of major intrinsic proteins which share weak similarities with previously identified aquaporin subfamilies was recently identified in land plants, and named X (for unrecognized) intrinsic proteins (XIPs). Because XIPs are still ranked as uncharacterized proteins, their further molecular characterization is required. Herein, a systematic fine-scale analysis of XIP sequences found in flowering plant databases revealed that XIPs are found in at least five groups. The phylogenetic relationship of these five groups with the phylogenetic organization of angiosperms revealed an original pattern of evolution for the XIP subfamily through distinct angiosperm taxon-specific clades. Of all flowering plant having XIPs, the genus *Populus* encompasses the broadest panel and the highest polymorphism of XIP isoforms, with nine *PtXIP* sequences distributed within three XIP groups. Comprehensive *PtXIP* gene expression patterns showed that only two isoforms (*PtXIP*2;1 and *PtXIP*3;2) were transcribed in vegetative tissues. However, their patterns are contrasted, *PtXIP*2;1 was ubiquitously accumulated whereas *PtXIP*3;2 was predominantly detected in wood and to a lesser extent in roots. Furthermore, only *PtXIP*2;1 exhibited a differential expression in leaves and stems of drought-, salicylic acid-, or wounding-challenged plants. Unexpectedly, the *PtXIPs* displayed different abilities to alter water transport upon expression in *Xenopus laevis* oocytes. *PtXIP*2;1 and *PtXIP*3;3 transported water while other *PtXIPs* did not.

Key words: Aquaporin, evolution, in situ hybridization, molecular physiology, *Populus*, XIP.

Introduction

Water flux across biological membranes can occur directly through the lipid bilayer, but predominantly happens through water channel proteins named aquaporins (AQPs) (Agre \textit{et al.}, 1993). AQPs are integral membrane proteins of the larger major intrinsic protein (MIP) superfamily. These channels are found in all living organisms, from archaea and eubacteria to fungi, animals, and plants (Engel and Stahlberg, 2002). Looking at this global distribution, AQPs are...
especially abundant in plants, with expression in virtually all cell types (Kjellbom et al., 1999; Wallace and Roberts, 2004). AQPs constitute a large and divergent family with 35 members identified in Arabidopsis thaliana (Johanson et al., 2001), 36 in Zea mays (Chaumont et al., 2001), 33 in Oryza sativa (Sakurai et al., 2005), 37 in Solanum lycopersicon (Sade et al., 2009), and 65 members in Populus trichocarpa (Gupta and Sankararamakrishnan, 2009). Some AQPs form channels facilitating bidirectional water fluxes, whereas others can conduct a wide range of small neutral solutes including urea, lactic acid, glycerol, hydrogen peroxide, silicic acid, metalloids, and gases such as ammonia and carbon dioxide. AQPs play a central role in plant–soil water relations, seed germination, cell elongation, drought resistance, salt tolerance, and fruit ripening. Based on sequence similarities, AQPs of most plant species can typically be divided into four subfamilies: the plasma membrane intrinsic proteins (PIPs), the tonoplast intrinsic proteins (TIPs), the nodulin 26-like intrinsic proteins (NIPs), and the small basic proteins (PIPs), the tonoplast intrinsic proteins (TIPs), the divided into four subfamilies: the plasma membrane intrinsic proteins (PIPs), the tonoplast intrinsic proteins (TIPs), the nodulin 26-like intrinsic proteins (NIPs), and the small basic intrinsic proteins (SIPs). Most of these subfamilies are being extensively investigated with respect to their structural signatures, expression patterns, subcellular localizations, and substrate specificities. Phosphorylation, pH, Ca²⁺, and osmotic gradients were also reported to affect their water channel activities. Moreover, recent insights into their co- and post-translational modifications and cellular trafficking have led to promising models that explain the regulation of their transport activity in various physiological processes, including photosynthesis, osmotic regulation, tree embolism recovery, and water use efficiency. Recent reviews have summarized increasing knowledge about these processes (Chaumont et al., 2005; Kaldenhoff and Fischer, 2006; Maurel, 2007; Maurel et al., 2009).

Three additional AQP subfamilies were recently described in the non-vascular moss Physcomitrella patens: GlpF-like intrinsic proteins (GIPs) homologous to some glycerol channels of Gram-positive bacteria (Gustavson et al., 2005), the hybrid intrinsic proteins (HIPs), and un categorized members designated X intrinsic proteins (XIPs) (Danielson and Johanson, 2008). Members of the latter subfamily are found in protozoa, fungi, and land plant kingdoms. Since XIPs are not encoded by the sequenced genomes of certain plants such as Arabidopsis, Oryza or Zea, partial knowledge together with hypothetical functions of plant XIPs are starting to emerge (Danielson and Johanson, 2008; Sade et al., 2009; Gupta and Sankararamakrishnan, 2009; Shelden et al., 2009; Park et al., 2010; Bienert et al., 2011). Plant XIP sequences have substantially diverged from those of fungi and protozoa. Strikingly, such sequence divergence is encountered even within the plant kingdom itself, as observed when comparing the Lycopodiophyta Selaginella moellendorfii, the Bryophyta Physcomitrella patens, and various Magnoliophyta (angiosperms) XIP sequences. Within this last plant division, the XIP subclass seems to be exclusive to Magnoliopsida (dicots) and its members have been phylogenetically clustered into two groups (XIP1 and XIP2). As these proteins harbour amino acid variations in regions corresponding to the primary selection filter and the major checkpoint for solute permeability [i.e. the first Asn-Pro-Ala (NPA) box and aromatic/arginine region (ar/R)] (Beitz et al., 2006; Tornroth-Horsefeld et al., 2006; Mitani-Ueno et al., 2011), it was first hypothesized that XIPs are non-functional as water channels but instead are dedicated to the transport of hydrophobic solutes (Danielson and Johanson, 2008; Gupta and Sankararamakrishnan, 2009). These predictions have been recently validated by Bienert and his collaborators (2011) on XIPs from three Solanales: Nicotiana tabacum, Solanum lycopersicon, and Solanum tuberosum.

Although substantial progress has been made regarding the XIP molecular structure, subcellular localization, transcriptional regulation, and functional gating, the reason for their availability in plants is not understood, and several questions remain to be answered. For instance, (i) to what extent did the evolutionary expansion of XIP occur in plants? The evolutionary history of the XIPs was partially explained in a broad phylogenetic survey including some sequences of flowering plants (angiosperms), protozoa, fungi, and mosses (Gupta and Sankararamakrishnan, 2009). This was paradoxically done without considering the angiosperm evolutionary story and this Viridiplantae division encompasses the largest number of XIP sequences. It follows that previous XIP phylogenetic data alone were not conclusive. (ii) Can some XIPs differ from those studied in Solanales be considered as multifunctional channels, being able to transport water? Indeed, many members in each MIP subclass present different selectivity for substrates, and this is particularly true in the case of plants which comprise the most remarkable MIP diversity.

In the study, XIP sequences from a wide variety of plants were first retrieved and compared, making full use of the available expressed sequence tag (EST) as well as plant genome sequences databases. This data set was used to analyse their phylogenetic relationship, together with an integrative amino acid-based evolutionary partitioning of this subclass during angiosperm evolution. Among this set, poplar, with nine sequences, is to date the species with the largest number of XIPs with significant amino acid diversity, making it a suitable model to re-examine this AQPs subclass molecularly. Expression analyses using quantitative real-time PCR revealed that only two PtXIP genes (PtXIP2;1 and PtXIP3;2) were expressed in vegetative tissues, and only PtXIP2;1 was differentially and strongly modulated in response to abiotic stresses. Supplementary in situ hybridization experiments showed that the PtXIP2;1 and PtXIP3;2 genes were highly expressed in well-defined cell types or poplar tissues. Finally, PtXIP2;1 and PtXIP3;3 were functionally characterized as being the poplar XIPs able to transport water in Xenopus oocyte expression assays.

**Materials and methods**

**Plant material and experimental design**

*Populus trichocarpa* (Torr. & Gray ex Hook), clone INRA 101-74 kindly provided by Dr Catherine Bastien (INRA, Orléans, France), was selected for the experiments as it is increasingly being
used as the male parent for genetic research programmes. Homoge-
neous 25 cm long woody stem cuttings were planted in 10.0 l pots
filled with a commercial substrate (40% black, 30% brown, and 30%
blond peat moss, pH 6.1, DUMONA-RN 75-3851 Arandon, The
Netherlands) and placed in a controlled-environment greenhouse
under a 16 h/8 h light/dark photoperiod, at 18/22 °C (day/night),
with relative humidity set at 70±10% and a daytime photosynthetic
photon flux ≥350 μmol m⁻² s⁻¹. Three treatments were conducted on
3-month-old plants. Phytohormone treatment was carried out on
fully expanded leaves with 1 mM salicylic acid (SA) dissolved in
100 μl of absolute ethanol before concentration adjustment in
distilled water and then pulverization. Drought conditions were
produced by withholding water until the first basal leaves showed
signs of severe dehydration (soil water potential Ψₛ ≥ −1.2±0.15
MPa). Rehydration conditions were obtained by re-watering the
soil. For mechanical woundings treatments, 40% of the leaf area was
lightly crushed using large clamps. At each sampling time, harvested
tissues were immediately frozen in liquid nitrogen and stored at
−80 °C until RNA extraction.

Bioinformatics analysis
The plant XIP gene subfamily was explored using XIP sequences
previously described (Danielson and Johanson, 2008; Gupta and
Sankararamakrishnan, 2009) as initial queries. Basic Local Align-
ment Search Tool (BLAST) search tools, tBLASTn and BLASTp
(Altschul et al., 1997), were used against Viridiplantae taxon non-
redundant generalist databases National Center for Biotechnology
information (NCBI, http://www.ncbi.nlm.nih.gov/) and Depart-
doe.gov/), and from plant-dedicated assembly genome projects:
Coffee Genome Project (http://www.lge.ibi.unicamp.br/cafe), Ge-
nome Database for Rosaceae (http://www.rosaceae.org), Genoscope
(http://www.genoscope.cns.fr), Public Eucalyptus Genome resource
(http://eucalyptusbdb.bi.up.ac.za), and Sol Genomics Network
(http://solgenomics.net). Incomplete sequences showing conserved
regions were manually assembled for further analysis, whereas
redundant entries, including sequences with putative point muta-
tions or polymorphism (similarities >98%), and single incomplete
sequences displaying a hypothetical length <80% compared with its
putative poplar orthologue were discarded.

The poplar MIP gene family was uncovered via two bioinfor-
matics approaches: P. trichocarpa whole genome shotgun (WGS)
sequences expanded with GenBank Populus EST collections and
full-length cDNAs, and the recent release of the P. trichocarpa genome
from JGI database assembly version 2.2 (JGI v2.2 annotation of
the v2.2 assembly (JGIv2.2) available via Phytozone version 7.0 (www.
phytozone.net)) in comparison with the older JGI annotation of the
v1.1 assembly (JGIv1.1). A complementary analysis was carried out on
the Molecular Genbank non-redundant sequence databases using the
tBLASTn search tool server in order to identify potential poplar
ESTs related to each MIP sequence previously identified. This made
it possible to redefine any sequence with false predictions. The
nomenclature of Gupta and Sankararamakrishnan (2009) was used for
naming Populus MIPs, except for the PtXIP subfamily, for which
an updated nomenclature was proposed based on the percentage of
amino acid similarity between members and the phylogenetic studies
presented herein.

Percentages of amino acid similarity and identity were calculated
using the NCBI blast2seq algorithm. Multiple sequence alignment
analysis was performed using PRALINE-PSI (Simossis and Herina,
2005) with a five PSI-BLAST iteration search (with an e-value cut-
off of 0.01) within the non-redundant database. Jalview (Clamp
et al., 2004) was used with the ClustalX colour code to illustrate the
alignment results.

The unrooted phylogenetic trees were constructed using the
maximum likelihood (ML) method implemented in the PhyML
program v3.0 (Guindon and Gascuel, 2003). Specifically, PhyML
analyses were conducted with the Jones–Taylor–Thornton (JTT)
substitution matrix, 1000 bootstrap replicates to assess the reliability
for degree of support for each internal branch on the phylogenetic
trees, estimated proportion of invariable sites, four rate categories,
and estimated gamma distribution parameter. Trees were viewed
and edited with TreeDyn (Chevenet et al., 1997), and bootstrap
values >50% were reported. Except for PtXIP3b (ProteinID
829126), all other related pseudogenes and truncated amino acid
sequences were eliminated from further analysis.

Total RNA isolation, cDNA synthesis, and amplification
Total RNA was extracted from 150 mg of roots, the apical part of
the stem, bark (including phloem) and wood from the intermediate
part of the stem, buds, and leaves using cetyltrimethylammonium
bromide (CTAB) extraction buffer according to Chang et al.
(1993). First-strand cDNA was synthesized from 1 μg of total RNA
using SuperScript III (Invitrogen, Carlsbad, CA, USA) following the
manufacturer’s instructions. Real-time PCR amplifi-
cation was performed using a MyiQ thermocycler (Bio-Rad,
Hercules, CA, USA) with MESA GREEN qPCR MasterMix Plus
(Eurogentec, Belgium) containing 2 μl of 40-fold diluted cDNA
according to the manufacturer’s protocol. The thermal profile of
the reaction was: 94 °C for 3 min and 40 cycles of 94 °C for 20 s,
54–58 °C for 20 s, and 72°C for 20 s. The specificity of amplicons
was routinely verified by melting curve analysis, and checked by
gel electrophoresis. PCR efficiencies were deduced for each gene
according to Pfaffl’s (2001) procedures. The crossing cycle number
(Cₚ) was automatically determined for each reaction by the iCycler
IQ v2.0 software with default parameters. For normalization of
the target gene abundance, the software application BestKeeper
v1 (http://www.gene-quantification.info; Pfaffl et al., 2004) was used
according to the developers’ manual to determine the best suited
reference genes from nine widely used housekeeping genes
(Czechowski et al., 2005; Xu et al., 2011), and then to estimate a
BestKeeper Index. The reference genes selected (gene locus; GM,
geometric mean of Ct ± SD; CV, BestKeeper coefficient of Pearson
correlation; and r, variance from all sample at all time points) were
Actinal (POPTR_0001s31700; GM, 24.78±0.59; CV, 0.240; r, 0.864),
SAND (POPTR_0009s01980; GM, 29.74±0.59; CV, 1.99; r, 0.870),
and TIP41α-like (POPTR_0001s30580; GM, 27.12±0.77; CV,
2.66; r, 0.787), chosen from different protein families in order to
reduce the possibility of co-regulation. Constitutive MIP transcript
levels in AtXIP1a and constitutive and drought induced levels of PtXIP
were assessed by comparison of the mean of the Ct after normalization with the
BestKeeper Index. The final results from each statistical output
were graphically represented after distribution and assignment of
a score value between 0 and 100, an arbitrary scoring range. A Ct
of 20 was assigned an arbitrary value of 100 (corresponding to the
highest MIP expression level in Populus), while a Ct of 40 was
assigned an arbitrary value of 0 (no expressed gene). Then all other
Cts were assigned values between 0 and 100, scaled based on their
relative distributions. As for differential accumulation of MIP
transcripts in response to stresses, the relative changes were
calculated with the equation 2⁻ΔΔCt according to Pfaffl procedure’s
(2001). Circadian PtXIP expression under control conditions was
first monitored allowing t₀ untreated samples to be used as controls
(corresponding to leaves sampled just before experiments). For
legibility, and especially for down-regulated genes, values were
graphically normalized to 0, this course representing no change in
gene expression. Each unit on both sides of 0 corresponds to a 2-fold
increase and a 2-fold decrease. The mean Ct value was determined
from three independent biological replicates for each sample, and
every PCR was carried out in triplicate. Values are given as means
± SD. Primers were designed using the Primer3plus application
(http://www.bioinformatics.nl/primer3plus; Rozen and Skaletsky,
2000), and are listed in Supplementary Table S3 available at JXB
online. A concomitant analysis of genomic DNA and cDNA was
carried out in order to evaluate the robustness of each primer set
(data not shown).
In situ hybridization

Fresh stems and leaves were harvested, cut, and immediately fixed in 4% (v/v) paraformaldehyde + 2.5% (v/v) glutaraldehyde overnight at 4°C. Fixed samples were dehydrated and embedded in methacrylate resin (methyl methacrylate–butyl methacrylate resin, EMS, Mülndehlheim, France) according to the manufacturer’s instructions. Polymerization occurred in gelatin capsules overnight at 60°C. Transverse sections of 3–4 μm thickness were cut with a rotary microtome, mounted on SuperFrost Plus slides (Fisher Scientific, Elancourt, France), and dried at 42°C for 2 d. A 5 min incubation in pure acetone removed the methacrylate resin. In situ hybridization (ISH) was performed as described in Leblanc-Fournier et al. (2008). Gene-specific RNA probes were designed to be located in the variable 3′-untranslated region (UTR) of P/XP2;1 and P/XP3;2 transcripts with an average size of 250 ribonucleotides (primers listed in Supplementary Table S3 at JXB online). DNAs encoding the probes were cloned in pGEM® T-Easy vector (Promega, Madison, WI, USA). Briefly, sense and antisense digoxigenin (DIG)-labelled RNA probes were synthesized as described in Leblanc-Fournier et al. (2008) using an in vitro transcription kit [DIG RNA labeling kit (SP6/T7), Roche Diagnostics, Mannheim, Germany] according to the manufacturer’s instructions. The antisense and sense probes were transcribed from SP6 or T7 polymerase promoters (after vector linearization with EcoI or SpeI, respectively). Stem and leaf sections were incubated overnight at 50°C with 1.5 ng μl⁻¹ or 3 ng μl⁻¹, respectively, of sense and antisense probes. They were then washed with 2× SSC/50% formamide. Detection was performed using anti-digoxigenin–alkaline phosphatase conjugate, followed by colorimetric detection of phosphatase activity (Bio-Rad). After suitable colour development, the reaction was stopped by rinsing in water, and sections were dried and mounted in Eukitt (Euromedex, Mülndehlheim, France). Observations were performed under an Axioskop 2 microscope (Zeiss, Jena, Germany). Data were recorded on a digital camera (Axiocam HR, Zeiss) using Axiosvision digital imaging software.

Expression in Xenopus laevis oocytes and osmotic water transport assay

The P/XP eCDNs cloned in a pGEM®-T Easy vector were amplified by PCR (primers listed in Supplementary Table S3 at JXB online). Upstream of each P/XP primer specific for the 5′-UTR and 3′-UTR, the BglII and SpeI restriction sites were introduced, respectively. The ampiclons were digested by BglII and SpeI (Promega) and the resulting fragments were cloned in the corresponding sites of a T7Ts vector, fused with the 5′- and 3′- UTR sequences of a Xenopus #2-globin gene, to favour stability of the derived complementary RNA (cRNA). The integrity of the two constructs was checked by sequencing. Plasmids were linearized by EcoRI before in vitro transcription, and functional expression of aquaporins in X. laevis oocytes was as described by Maurel et al. (1993). The statistical analysis was performed on data pooled from all experiments using a one-way analysis of variance (ANOVA; \( P < 0.001 \)), and post-hoc multiple comparisons were run using a Tukey test on the same set of data for which normality was checked. In the figures, the bars indicate the SEM and different letters denote statistically significant (\( P < 0.001 \)) differences.

Results

Phylogenetic analysis of the angiosperm XIP group

An evolutionary lineage fine analysis of all available angiosperm XIP-like proteins was performed, based on the current angiosperm phylogeny. To accomplish this, all XIP sequences previously reported by Danielson and Johanson (2008) and by Gupta and Sankararamakrishnan (2009) were used as queries against various genomic and EST angiosperm collections. From slightly less than 200 XIP-related sequences retrieved, 55 non-redundant representative sequences were selected (Supplementary Table S1 at JXB online). As previously mentioned, except for Liriodendron tulipifera (Magnoliidae), all XIP sequences came from the dicotyledonous phylum. In addition, an unrooted tree constructed from alignments of protein sequences confirmed that XIPs split into two independent clusters, identified as XIP-A and XIP-B in this study (Fig. 1; sequence alignments in Supplementary Fig. S3). These two subgroups correspond to XIP2 and XIP1, respectively, in the former nomenclature (Gupta and Sankararamakrishnan, 2009). A fine reading of the phylogenetic XIP distribution led to further refinement of the former nomenclature. The XIP-A cluster encompassed 15 orthologous sequences from mesangiosperms (Magnoliid superorder with L. tulipifera), and various core eudicots within the Rosanae taxon exclusively. These XIP-A sequences shared an average amino acid similarity of 77%, which fell to ~60% with XIP-B members. The second cluster, XIP-B, reflected the emergence of four major clades: XIP2, XIP3, XIP4, and XIP5 with well-supported to strong bootstrap support (82, 95, 99, and 95%, respectively). Interestingly, XIP-Bs were exclusive to eudicots and, unlike XIP-As, distinctly clustered into plant taxon-specific clades. Thus, XIP2 and XIP3 were spread into the Rosidae superorder: XIP2 encompassed sequences from Malpighiales (Euphorbia, Gossypium, Manihot, Populus, and Ricinus), Brassicales (Carica), and Rosales (Malus and Prunus), while XIP3 members were encountered in Malpighiales (Manihot and Populus) and Sapindales (Citrus). The XIP4 and XIP5 clades were, in contrast, exclusively linked to the Asteranae superorder: XIP4 members were linked to Lamids (Ipomea, Mimulus, Nicotiana, Solanum, and Triphysaria), while XIP5 members were linked to Campanulids (Centauraea, Cichorium, Helianthus, Guizotia, and Lactuca). Members within each XIP-B clade shared a minimum of 85% amino acid sequence similarity, and an average between-clade similarity of 79%. Finally, as reported for the monophyletic Lilipopsida taxon (Danielson and Johanson 2008), if the absence of XIP sequences is true and not an artefact due to limited data sets, plants belonging to ferns, ANITA (the basal angiosperms, Amborella, Nymphaeales, Illiciales, Trimeniaceae, and Austrobaileya), and coniferophyte monophyletic taxa lack the XIP subfamily.

A more integrative view of this phylogenetic XIP clade distribution (Fig. 2) was also revealed when considering the current angiosperm phylogeny (Angiosperm Phylogeny Website, V.9 http://www.mobot.org/MOBOT/research/ APweb/, complemented with the Angiosperm Phylogeny Group III system, Chase and Reveal 2009). This analysis showed a XIP-A branch in which members diverged parallel to the plant species divergence, whereas the XIP-B branch would reflect clade diversifications in a plant taxon-specific manner, both with their own evolutionary rate.

Features of the XIP gene subfamily in Populus

The molecular characterization of the Populus XIP-like genes was updated using the XIP protein sequences retrieved from
a reiterative search against the Phytozone Version 7.0. This upgraded version includes the last JGI v2.2 annotation of the *P. trichocarpa* assembly (JGIv2.2). The opportunity to retrieve the whole poplar *MIP* gene complement and to re-examine their sequences was also taken. Sixty-five sequences are by default annotated as putative ‘*Aquaporin (major intrinsic protein family)*’, leading to 54 full-length open reading frames (ORFs) as initially reported (Gupta and Sankararamakrishnan, 2009, Almeida-Rodriguez et al, 2010). This research has led to the retrieval of two other sequences belonging to the XIP subfamily: *PtXIP1;2* (POPTR_0009s13105) and a pseudogene at the nucleotide position 10 525 867–10 526 066 located on the scaffold IX and not annotated. Sequences and final Phytozome v7.0 labelling are given in Supplementary Table S2 at JXB online. In agreement with previous works, the 56 ORFs

Fig. 1. Evolutionary relationship between Viridiplantae plant XIPs. The unrooted phylogeny of the XIP protein sequence was inferred using maximum likelihood. The tree was produced using PhyML with a genetic distance calculated by the JTT model of amino acid change. The numbers at the nodes represent the percentage bootstrap values (>50%) based on 1000 reassembling. The distance scale denotes the number of amino acid substitutions per site. Green circled numbers represent the percentage amino acid sequence identity and similarity intragroup; other numbers are amino acid sequence identity and similarity intergroups. *Populus* XIP sequences are highlighted in red. Species and accession numbers are listed in Supplementary Table S1 at JXB online. Selaginella moellendorffii and *Physcomitrella patens* XIP sequences (XIP*) were edited for information purposes, and were used as angiosperm taxon outgroups. *Populus* XIP aquaporins | 2221
were phylogenetically assigned to five distinct subfamilies (phylogenetic distribution and sequence alignments detailed in Supplementary Figs S1 and S2): PIPs (with 15 members subdivided into five PIP1s and 10 PIP2s), TIPs (17 members), NIPs (11 members), and SIPs (six members). For consistency, gene names specified by Gupta and Sankararamakrishnan (2009) were used for this work. These groups will not be discussed further. The fifth subfamily defines the as yet uncharacterized XIPs, and constitutes the main issue of the present discussion.

The poplar XIP subfamily appears to comprise nine putative members with seven full-length protein sequences (DbXrefJGI 579650 and PtXIP-nd). The 829126 sequence (PtXIP3:1b) was a truncated sequence due to an ATA triplet insertion within the coding sequence, generating a stop codon nine bases downstream of the initiation codon in relation to its paralogue 557139 (PtXIP3:1). This insertion was validated by sequencing multiple clones.

Finally, the full-length PtXIP-related proteins were classified into three groups: PtXIP1 (with two members: PtXIP1:1-557138 and PtXIP1:2-POPTR_0009s13105), PtXIP2 (with one member: PtXIP2:1-821124), and PtXIP3 (with three members: PtXIP3:1-557139, PtXIP3:2-767334, and PtXIP3:3-759781). This new nomenclature is supported by the following three arguments.
(i) Conventionally, plant aquaporins are divided into subfamilies (i.e. NIPs, PIPs, SIPs, and TIPs) that are further divided into groups of related proteins. To maintain consistency with the AQP nomenclature principles of Johanson et al. (2001), the PtXIP subfamily should be divided into three groups. Additionally, this repartition was confirmed by the phylogenetic distribution of PtXIPs alone (Fig. 3A). Proposed names for PtXIPs consisted of the subfamily name followed by a number indicating the XIP master group and a second number characterizing the individual XIP member within the group. Intergroup amino acid sequence similarities between PtXIP1 and PtXIP2 or PtXIP3 were ~59%, and ~81% between PtXIP2 and PtXIP3 (Fig. 3B). PtXIP1 members were highly conserved, with a similarity of 94%, as well as PtXIP3s with an average of 90% similarity. The pseudogene sequences and the truncated protein 829126-related sequence (PtXIP3;1b in Fig. 3) were not classified, although they seem to be paralogues of PtXIP2;1 and PtXIP3;1, respectively.

(ii) PtXIP1;2 was distinguished by a gene structure with a unique intron, whereas PtXIP1;1 was an intronless gene. Both exhibited a longer N-terminus as compared with other MIP homologues, a hydrophilic C-loop region deleted of eight amino acid residues specific to the dicot XIP subfamily, and the substitution of the first NPA motif by an SPV motif (Supplementary Fig. S3 at JXB online). This substitution was also encountered in Lotus and Glycine max sequences, while the other XIP1 clustered sequences showed a substitution of the alanine residue of the NPA motif by a valine, isoleucine, or cysteine residue. Remarkably, PtXIP2;1 had both NPA motifs strictly conserved.

(iii) Lastly, the evolutionary relationships of the poplar PtXIPs in a compilation of all the angiosperm XIP-like proteins characterized so far backed up overall the choice of nomenclature (Fig. 1).

The new JGIv2.2 nomenclature showed that Populus MIP genes were evenly spread over 18 of the 19 haploid ‘chromosomes’ constituting the poplar genome (Supplementary Table S2 at JXB online). Yet, seven out of the nine PtXIP sequences were arranged head-to-tail on linkage group IX (in order, and taking the new nomenclature into account: PtXIP2;1–PtXIP1;2–PtXIP3;1b–PtXIP-nd–PtXIP1;1–PtXIP3;1–PtXIP3;2), one was located on linkage group IV (PtXIP3;3), and the final one (PtXIP-579750) was located on a scaffold that had not yet been assigned to a specific linkage group (Fig. 3C). Such scaffolds were reported to be heterochromatic or derived from substantially divergent haplotypes in the sequenced clone (Tuskan et al., 2007; Kelleher et al., 2008).

Expression pattern of PtXIP transcripts

The transcriptional expression of the PtXIP gene subfamily and two PtPIP genes as references, PtPIP1;2 (mentioned as...
PtPIP1;1 in Secchi et al., 2009) and PtPIP2;2, was traced in various vegetative organs and in plants exposed to different stresses. The steady-state level of constitutive transcript accumulation relative to the XIP subfamily was monitored in different vegetative tissues including dormant buds (apical and axillary buds mixed), leaves at different maturation stages, apical growing stems, wood and bark (including phloem) of 2-year-old mature stems, and roots (Fig. 4). Of the nine members, only PtXIP2;1 and PtXIP3;2 showed detectable expression in P. trichocarpa 101-74. No transcripts of PtXIP1 genes PtXIP3;1, or PtXIP3;3 were detected. PtXIP2;1 was ubiquitously expressed in vegetative tissues, with high accumulation in buds and immature organs (stems and leaves). It decreased significantly in mature leaves and dropped drastically in senescent leaves. A moderate transcript accumulation was monitored in roots. As regards PtXIP3;2, its expression was high in wood, and to a lesser extent, in buds and roots. In all other organs, PtXIP3;2 expression was very low (mature leaves and bark) or not detectable. PtXIP1 genes, PtXIP3;1, and PtXIP3;3 did not show significant transcript accumulation under the observed conditions. PtPIP1;2 and PtPIP2;2 were highly expressed in all the vegetative tissues of P. trichocarpa studied.

PtXIP2;1 and PtXIP3;2 gene expression was assessed in leaves and branches of plants subjected to severe drought followed by soil re-watering when the most basal leaves showed signs of physiological dehydration. Expression of PtXIP genes was also monitored in leaves exposed to SA or mechanical wounding (Fig. 5). Regardless of treatments, PtXIP2;1 showed a marked transcriptional modulation, whereas PtXIP3;2 showed limited transcriptional modulation including in stems where its constitutive accumulation appears substantial. No circadian variations in PtXIP mRNA expression patterns were observed (data not shown). During water stress and re-watering (Fig. 5A), PtXIP2;1 gene expression was greater in leaves than in stems. PtXIP2;1 was quickly and drastically down-regulated in response to drought. Interestingly, PtXIP2;1 expression increased 30 min after induction by re-watering and then dropped to its lowest level at 6 h.

Gene expression recovered to the basal level 12 h after re-watering. Under SA and wounding treatments, transcripts of PtXIP2;1 were transiently up-regulated (Fig. 5B, C). Although modulation occurred earlier under SA than wounding, transcripts peaked at 12 h and returned to a steady-state expression level at 24 h in both cases.

Similar to PtXIP2;1, PtPIP modulations in response to drought were more marked in leaves than in stems. PtPIP1;2 transcript abundance showed an early and marked up-regulation, whereas PtPIP2;2 transcript abundance was down-regulated. Both returned to basal levels only 6 h after re-watering. Lastly, transcript levels of PtPIP1;2 were down-regulated by SA and transiently up-regulated by wounding, whereas transcript levels of PtPIP2;2 were up-regulated by both SA and wounding.

**In situ hybridization**

ISH experiments performed on cross-sections of non-stressed *Populus* stems and leaves showed that PtXIP2;1 transcripts were highly abundant in most vegetative tissues (Fig. 6A–D, 1–N), while PtXIP3;2 expression seemed to be restricted to stems (Fig. 6E–H; Supplementary Fig. S4 at *JXB* online). In stems, PtXIP2;1 expression was detected in bark, phloem, and wood with a relatively high intensity (Fig. 6A, C). PtXIP3;2 transcripts co-located well with those of PtXIP2;1 (Fig. 6E, G). In leaves, due to the cell turgor level and a predominantly vacuolar cell volume, coloration is located in the cell periphery. PtXIP2;1 expression was detected in almost all parts of the midrib except the cambium (Fig. 6I). In the lamina (Fig. 6K, M) PtXIP2;1 was present in the epidermis, spongy parenchyma, and vascular tissues of lateral veins (Fig. 6M, arrow lv).

**Water transport properties of PtXIPs**

To gain further insights into the function of PtXIPs, cRNAs encoding the related proteins were transcribed *in vitro* and injected into *Xenopus* oocytes. After 2–3 d, oocytes were

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**Fig. 4.** Real-time quantitative RT-PCR analyses of the constitutive PtXIP2;1 and PtXIP3;2 transcript accumulation in vegetative tissues. The expression level of PtActin 1, PtSAND, and PtTIP41-like gene was used as the normalization internal control. PtPIP1;2 and PtPIP2;2 expression was followed for comparison purposes. Bars represent standard deviations of at least three technical repetitions from three independent biological experiments. Four plants were pooled for this biological analysis.
Fig. 5. Real-time quantitative RT-PCR analyses of PtXIP2;1 and PtXIP3;2 transcript accumulation kinetics in stress-challenged plants: (A) in leaves and intermediate parts of the stems during the drought stress time-course followed by recovery (re-watering noted at time 0), (B) in 1 mM SA-treated leaves, and (C) in wounded leaves. The expression level of PtActin 1, PtSAND, and PtTIP41-like genes was used as the normalization internal control. PtPIP1;2 and PtPIP2;2 genes were followed for comparison purposes. Bars represent the standard deviations of at least three technical repetitions from three independent biological experiments. Three plants were pooled per biological assay.

transferred into an hypo-osmotic solution and their osmotic water permeability ($P_t$) was deduced from the swelling kinetics of individual oocytes. Oocytes that did not receive any cRNA or that expressed the highly active aquaporin PtPIP2;2 were taken as negative and positive controls, respectively. Figure 7 shows that whereas injection of PtXIP1s, PtXIP3;1, and PtXIP3;2 cRNA did not induce any change in oocyte $P_t$, expression of PtXIP2;1 and PtXIP3;3 increased $P_t$ by $\sim$2.5-fold. In parallel experiments, oocytes expressing PtPIIP2;2 showed an even more pronounced ($\sim$8-fold) increase in $P_t$. The standard errors are very small because of the high number of cell replicates, which is much higher than in other similar studies. Checks were carried out to verify that similar results (i.e. enhanced $P_t$ in PtXIP2;1 oocytes) were obtained in several independent oocyte batches and cRNA preparations. The present data establish a significant water channel activity for PtXIP2;1 and PtXIP3;3 but not for PtXIP1s, PtXIP3;1, and PtXIP3;2.

Discussion

XIP diversification in the light of angiosperm evolutionary expansion

To date, the comparison of XIP sequences from fungi and plant phyla has been limited to residues that constitute loops and ar/R selectivity filters, and to exon–intron organization (Gupta and Sankararamakrishnan, 2009). In this previous work, it was asserted that during evolution amino acids of the ar/R filter became more hydrophobic in dicot XIPs than in their homologues of fungi and moss. This could reflect a distinct spectrum of solutes transported by these XIPs, which would entail a notable evolutionary divergence between various living kingdoms. Although this previous study sketched interesting evolutionary features, it did not provide insights into the angiosperm taxon by itself which, however, encompasses the great majority of XIP members. By pointing to a notable sequence diversity in angiosperms, the present work highlights evolutionary dynamics of XIPs within this taxon. Without an a priori use of previously described XIP sequences, a new query against plant EST collections resulted in the retrieval of 55 putative XIP sequences. All came from a very wide range of flowering plants which, except for Liriodendron, were land dicotyledons plants. Yet, the most striking observation lies in the relationship between the phylogenetic XIP distribution and the currently established phylogeny of angiosperms (Figs 1, 2). The present data emphasizes a prospective evolutionary scheme where XIP members clustered into at least five significantly distinct groups, including four which paralleled the divergence of angiosperm taxa. Although it is likely that the XIPs from angiosperms have originated from a common ancestral gene and have duplicated early after the emergence of this plant clade, it appears clearly that they have then substantially diverged into two clusters (XIP-A and XIP-B). The XIP-A (or XIPI) cluster might include the oldest angiosperm XIP sequences. Indeed, it encompasses the only sequences found in the early-diverging Magnoliales taxa. In core eudicots, except for a clade belonging to Rosanae (Fabibs) in which XIPIs are expressed, the XIPI evolutionary pathway marks genes for silencing in all other clades. Furthermore, no XIPI homologues appear in the Asteranae sister clade. As for the second XIP-B cluster, it represents the most interesting expanded subset, with at least four groups. As these groups are only found after core eudicot emergence, they may result from a more recent expansion compared with XIP-A. Thus, XIP2s and XIP3s are exclusively linked to the Rosanae superorder. XIP4s and XIP5s are represented exclusively in Asteranae plants. Finally, this discriminative organization contrasts with the homogenous PIP subfamily and the more heterogeneous NIP, TIP, and SIP subfamilies in which members do not split in such a plant taxa-classified manner.

Following gene duplication events, paralogous genes can take on different fates: this includes loss of some of paralogues, divergence and functional differentiation, or maintenance of partially overlapping functions (Conant and Wolfe, 2008). The various diversification scenarios displayed by the XIP subfamily offer a great opportunity to tackle four fundamental questions. (i) As XIP-A members showed a significant divergence and loss of expression in several core eudicots clades, would this branch be subject to loss of function over time? (ii) Would the XIP-B members be newly evolved sequences, suggesting specialized functions? (iii) Would natural selection pressure under XIP gene divergence ultimately be involved in speciation processes? (iv) Taken as a whole, despite the actual impossibility of
interpreting the presence of XIPs in numerous plant taxa which differ fundamentally (Judd et al., 2002; Soltis et al., 2004), could this unusual phylogenetic repartition be rationalized through some ecological and/or phenotypic explanations? Some of these questions will be answered once more extensive knowledge about ecological and phenotypic relationships, and larger genomic collections are available especially for plants belonging to early-diverging angiosperms and Asteranae.

The vastly expanded XIP subfamily is a unique feature of Populus species

Of the embryophytes analysed, poplar is unique in that it is the sequenced land plant that to date contains the highest amount and degree of diversity of XIP copies. The *P. trichocarpa* XIP subfamily (*Pt*XIPs) is composed of nine sequences: among which are six full-length coding sequences (CDS) [leaving aside two pseudogenes and one truncated...
Fig. 7. Osmotic water permeability ($P_i$) of oocytes expressing aquaporin isoforms. $P_i$ (± SE, with number of cells in parentheses) was measured in oocytes, injected either with water or with cRNAs encoding the indicated aquaporins. Data are from two representative experiments with different oocyte batches and cRNA preparations. Different letters above each bar represent statistically significant differences ($P < 0.05$; one-way ANOVA, Newman–Keuls test).

related product sequence ($PtXIP3;1b$). A fine analysis of the $PtXIP$ subfamily indicated that its members have phylogenetically diverged into three branches: $PtXIP1$ ($PtXIP1;1$ and $PtXIP1;2$), $PtXIP2$ ($PtXIP2;1$), and $PtXIP3$ ($PtXIP3;1$, $PtXIP3;2$, and $PtXIP3;3$).

An interesting outcome was revealed during AQP sequence compilation from the two JGI assembly versions: $PtXIP$s present a significant degree of polymorphism, contrasting with other subfamilies that showed less variation (Supplementary Table S2 at JXB online). As previously observed, XIP loci may result from high haplotype variations (Kelleher et al., 2007). Moreover, such amino acid substitutions can reveal potential adaptive evolutionary events, which generally come about with related pressure selection modalities (purifying, neutral, or positive/diversifying). Because the genetic code is redundant, any mutation may or may not be synonymous. Despite a limited number of Populus XIP-related ESTs (13 partial sequences phylogenetically homologous to $PtXIP2;1$ and four partial sequences homologous to $PtXIP3;1$), the ratio of non-synonymous versus synonymous substitutions was calculated ($\omega = dN/dS$) which is an indicator of the history of selection acting on a gene. Although expressed $PtXIP$s had high $\omega$ values ($PtXIP2;1:0.42$ and $PtXIP3;2:0.37$) compared with $PpPIP$s ($PpPIP1a:0.11$ and $PpPIP2b:0.05$), they remained <1. This suggests that in the course of their evolution, expressed $PtXIP$ genes underwent a purifying pressure selection, thus illustrating their functional role in the $Populus$ genus. A last argument in favour of such purifying pressure concerns the cluster organization of this XIP subfamily. Seven of the nine $PtXIP$ sequences are arranged head-to-tail on chromosome IX. Gene organization typically leads either to a tandem array of reiterated units (e.g. ($PtPIP2;5$)($PtPIP2;6$) or to a cluster (such as $PtXIP$ genes) when conversion and divergence events occurred (Graham 1995). Such a clustering feature is considered to facilitate the expansion in gene quantity through recombination, and may reflect an adaptive mechanism originating in genome-selective pressure and selection. However, following gene duplication events, paralogous genes can take on alternative fates (Conant and Wolfe, 2008). Within this $PtXIP$ cluster, the duplicated sequences $PtXIP3;1$ (expressed in $P. tremula$ and $P. tomentiglandulosa$) and $PtXIP3;1b$ (non-expressed) are an interesting case study of formation of a gene family in which paralogous copies differentially evolved. Indeed, rather than an acquisition of new adaptive functions through ‘positive’ mutation (neo-/subfunctionalization), $PtXIP3;1b$ may be incapacitated by the occurrence of a deleterious premature stop codon insertion (non-functionalization signature), and then becomes a functionless pseudogene.

A literature review highlights genetic redundancy as a salient feature of living organisms, and gene duplication events are considered as a primary driving force, providing raw material for evolutionary novelty (Taylor and Raes, 2004; Freeling, 2009; Kafri et al., 2009). However, besides the evidence that overlapping functions between duplicate genes manifest as synthetic aggravating interactions between paralogues, they could lead to major repercussions on related steady-state mRNA and/or protein pools and potentially on the regulatory mechanisms controlling various physiological processes, with far-reaching phenotypic effects (Gu et al., 2003; DeLuna et al., 2008). In the light of these data, it is intuitive to note that high non-synonymous variations and major mutations such as premature stop codons may reflect a significant excess of polymorphisms that substantially affect XIP protein structure and function. In other words, this supports and extends these evolutionary results showing that the $PtXIP$ loci and the plant XIP subfamily as a whole could be under a strong selection force associated with duplication and significant differentiations.

$PtXIP$ gene expression and related protein function assessments

Previous works have shown that, when over-represented in tandemly duplicated arrays, plant genes usually respond to environmental constraints (Hanada et al., 2008). Despite poplar XIP divergence and expansion, the expressed XIP genes are under-represented in poplar databases, with only 17 ESTs, and because most sequences were isolated from pooled tissues no general conclusion could be drawn regarding their expression pattern. XIP expression was evaluated first using microarray databases available in public repositories (Gupta and Sankararamakrishnan, 2009). Although microarray methods provide an unprecedented capacity for whole genome profiling, their limits are well characterized, and accurate normalization through quantitative PCR (qPCR) remains a somewhat unavoidable step for obtaining reliable and conclusive results. This is particularly true for the Affymetrix poplar genome array with only five XIP genes represented and for which related probesets data should be interpreted with substantial precaution. Here, XIP expression was reappraised in planta using qPCR. In addition, ISH was performed to determine cell-specific $PtXIP$ gene expression patterns. In accordance with expressed sequences
databases, only two members (PtXIP2:1 and PtXIP3:2) were found to be expressed in P. trichocarpa 101-74. Surprisingly, they differ substantially from each other by their own accumulative patterns. PtXIP2:1 was one of the most expressed poplar MIP genes. It was ubiquitously expressed in all vegetative tissues with a distinct developmental gradient. It peaked in leaves, with a marked cellular expression in vascular tissues, spongy parenchyma, and epidermis. To a lesser extent, PtXIP2:1 accumulated in wood, was uniformly distributed in bark and phloem, but was absent in the cambial region where cells have not reached a high level of specialization. As regards PtXIP3:2, it preferentially exhibited transcript accumulation in wood, and, although sublocalized in all part of stems (vessel-associated cells, xylem rays, bark, and phloem), its expression reached a maximum in xylem. Similarly, PtXIP2:1 and PtXIP3:2 were also expressed differently in challenged plants, where PtXIP2:1 showed the most contrasted transcript accumulation. A drastic down-regulation of PtXIP2:1 expression was observed under severe drought stress, whereas PtXIP2:1 was transiently up-regulated by SA and wounding. Furthermore, the oocyte expression experiments clearly revealed that PtXIP2:1 promotes a significant water channel activity. Considering that the plasma membrane localization of Solanales XIP could be extrapolated to PtXIP, these data may suggest different roles for XIPs in membrane transport at specific plant sublocalizations, and under stable or fluctuating environmental conditions. PtXIP2:1 could play a leading role in this, and, as suggested for PpPIPs and MIPs from various plant species (Heinen et al., 2009; Secchi and Zwieniecki, 2010; Almeida-Rodriguez et al., 2010; this study), PtXIP2:1 can be reasonably considered as a physiological co-actor contributing to regulate the cellular osmotic equilibrium.

Whereas the oocyte expression experiments did not allow any definitive conclusion about the putative transport activity of PtXIP1s, PtXIP3:1, and PtXIP3:2, this approach revealed a significant water permeability for PtXIP2:1 and PtXIP3:3. Previous studies have shown that several sequence regions within XIPs present notable differences compared with typical AQPss. Some of these concern substitutitions in the ar/R selectivity filter, forming a quite hydrophobic pore environment predominantly allocated to the conduction of hydrophobic and bulky solutes (Bienert et al., 2011). In the present assays and in line with its water transport functionality, only PtXIP2:1 clearly differs in sequence from the other PtXIPs, with a particular ar/R selectivity filter and canonical NPA boxes. This is also relevant comparing PtXIP2:1 with XIPs of other land plants. A significant water channel activity for PtXIP3:2 was also observed. This ability is quite puzzling as it was not possible to link it with any singular structural motif. Indeed, PtXIP3:3 harboured ar/R residues and NPA motifs similar to those of PtXIP1 and of other members of the PtXIP3 clade for which no water transport capacity was observed. These observations suggested that the transport water ability of PtXIP3:3 is not simply controlled by NPA motifs and the ar/R selectivity filter, and that other structural features could also be involved in this control. Further studies are needed to identify the residue(s) beyond those in the ar/R and NPA regions which are important for this water permeability. As for PtXIP1s, PtXIP3:1 and PtXIP3:2 were apparently devoid of water transport, and they exhibited several ar/R residue similarities with Solanales XIPs which suggested probable common channel activities. Their solute specificity will have to be established.

To conclude, XIP-related data illustrate the potential transport of alternative solutes between members from an MIP subclass, suggesting differential but complementary functional specialization in plant environmental adaptive responses. To conclude definitively on the water and solute transport functions of PtXIPs and their physiological relevance in a plant’s life processes, suppression of XIP gene function will have to be addressed in future experiments. Moreover, further studies are needed to determine the role, if any, of structural motifs in PtXIP-related functions and those involved in this XIP water permeability.

Supplementary data

Supplementary data are available at JXB online.

Figure S1. Phylogenetic trees of all the full-length members of the major membrane intrinsic AQP protein family of Populus trichocarpa cv. Nisqually.

Figure S2. Sequence alignment of all the full-length members of the major membrane intrinsic AQP protein family of Populus trichocarpa cv. Nisqually.

Figure S3. Protein sequence alignment of the 50 Viridiplantae XIPs.

Figure S4. In situ localization of PoptrXIP3;2 mRNA in leaves.

Table S1. List of the non-redundant representative Viridiplantae XIP gene sequences used in this work.

Table S2. Excel spreadsheet of the Populus trichocarpa cv. Nisqually MIP gene family including all available genomic annotations from JGI.

Table S3. Primers used for qPCR, in situ hybridization, and oocyte experiments.

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