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Functional analysis of putative genes encoding the PIP2 water channel subfamily in *Populus trichocarpa*.

FRANCESCA SECCHI[1], BRYCE MACIVER[2], MARK L. ZEIDEL[2], MACIEJ A. ZWIENIECKI[1]

[1] Arnold Arboretum of Harvard University, Cambridge, USA

[2] Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, USA

Corresponding author: Francesca Secchi; fsecchi@oeb.harvard.edu

Summary

We located fully sequenced putative genes of plasma membrane intrinsic proteins (PIPs) family in the *Populus trichocarpa* genome. Of 23 gene candidates, we assigned eight genes to the PIP2 subfamily. All eight putative genes were expressed in vegetative tissues (roots, leaves, bark and wood) and all showed water channel activity after being expressed in *Xenopus* oocytes. Six out of eight proteins were affected by mercury ions. No proteins were affected by the presence of nickel or tungsten ions, or by lowering pH of bathing external solution from 7.4 to 6.5. The presence of copper ions caused seven out of eight PIP2 proteins to increase their water transport capacity by as much as 50%. This systematic study of PIP2 subfamily of proteins in *P. trichocarpa* provides a basic overview of their activity as water channels and will be a useful reference for future physiological studies of plant water relations that use *P. trichocarpa* as a model system.

Introduction

Plant water relations are fundamental to the ability of plants to survive on land. The movement of water between cells is mediated by a large number of membrane water channels (aquaporins). In general, higher plant aquaporins are subdivided into five families based on sequence similarity: the plasma membrane intrinsic protein (PIPs), the tonoplast-intrinsic proteins (TIPs), the small basic intrinsic proteins (SIPs), the NOD-26-like intrinsic proteins (NIPs) and the most recently identified the uncategorized X intrinsic proteins (XIPs) (Danielson and Johanson 2008). PIPs represent the subfamily with the largest number of members and can be further divided into two subfamilies named PIP1 and PIP2. Differing in water permeability,

many PIP1 proteins have no water channel activity when expressed in oocytes, whereas PIP2 subfamily has high water permeability (Chrispeels et al. 2001). PIP2 proteins are currently the focus of intensive studies in many species, reflecting their importance for a variety of physiological functions (Agre et al. 1998; Aroca et al. 2007; Chaumont et al. 2001; Maurel 2007; Maurel and Chrispeels 2001; Tournaire-Roux et al. 2003; Tyerman et al. 1999; Ye and Steudle 2006). However, limited information exists for PIP2 subfamily aquaporins in the "woody plant" model system, including such basic information as the phylogeny of PIP2 gene candidates, their expression patterns, and their potential to function as water channels.

Here we report the results of a systematic *Populus trichocarpa* genome search for PIP gene candidates and their expression pattern in all major vegetative tissues including leaves, roots, bark, and wood. We also provide results of water channel activity of all eight PoptrPIP2 proteins using heterologous expression in *Xenopus laevis* oocytes. These experiments were combined with a search for ions with potential water channel gating function, with the goal of developing tools for future use in tissue or whole plant level physiological studies.

Materials and Methods

Plant materials and growth conditions

Populus trichocarpa cuttings were rooted in aerated hydroponic containers (6.5 L) filled with modified Hoagland solution (pH 6.00-6.50; 795 µM KNO_3, 603 µM $Ca(NO_3)$ 270 µM $MgSO_4$ and 109 µM KH_2PO_4; micronutrients: 40.5 µM Fe(III)-EDTA, 20 µM H_3BO_4, 2 µM $MnSO_4$, 0.085 µM $ZnSO_4$, 0.15 µM $CuSO_4$ and 0.25 µM Na_2MoO_4) and located in a growth chamber (18/21°C, 10 h day/night periods, 60% humidity with a 500 µmol photons m^{-2} s^{-1}

illumination). After two weeks, plants were transferred to 42 L boxes (12 plants per box) and the medium was replaced with low nitrate (5%) half Hoagland solution to promote root growth. Six-week-old plants were then transferred into 1 gallon pots filled with potting mix and placed in a greenhouse (12 h light/12 h dark cycle, 29/17 °C day/night). Plants were watered daily and natural daylight was supplemented with light from metal halogen lamps (500-600 µmol photons $m^{-2} s^{-1}$).

Tissue samples for expression analysis were collected from fully developed leaves, roots, bark (all tissues outside xylem) and wood around noontime. Tissue was immediately frozen in liquid nitrogen and stored at -80 °C.

Database search, sequences analysis and construction of neighbor-joining tree

The *P. trichocarpa* genome DNA Database was downloaded from DOE Joint Genome Institute web site (http://genome.jgi-psf.org/Poptr1/Poptr1.download.html).
The advanced text search function (gene annotations were already deposited) was used to find members of putative PIP family. Sequence analyses and comparison with known sequences were carried out using NCBI (National Centre of Biotechnology Information, USA) BLAST server. Multiple alignment amino acid analysis was performed with ClustalW program` (http://bioinformatics.ubc.ca/resources/tools/clustalx). Neighbor-joining tree was constructed using the neighbor-joining (NJ) method and drawn using MEGA4 (http://www.megasoftware.net/mega.html).

Total RNA isolation, cDNA synthesis and amplification

Total RNA was isolated from leaves, bark (including phloem), wood and roots according to the protocol of Chang (Chang et al. 1993). Acid nucleic was extracted from two independent biological groups, each group composed of two plants. Contaminant genomic DNA was removed from the samples by digestion with RNase-free DNase I (Fermentas), following the manufacturer's instructions. RNA integrity was checked by electrophoresis on 1% agarose gel and the concentration was quantified by measuring the absorbance at 260 nm. Five μg of RNA from each tissues was reverse transcribed to first strand cDNA using oligo-(dT)12–18 (Fermentas) as primer and SuperScript II Reverse Transcriptase (Invitrogen) according to manufacturer's instruction. To check DNA contamination in each sample, an additional control reaction was carried out in which the reverse transcriptase was omitted. cDNA and primers were tested by PCR, the amplification was performed using sufficient template amount, 500 nM of specific primers (the same used for Real Time PCR, described below) and AmpliTaq Gold polymerase (Applied Biosystems). The PCR cycles were: 1 cycle of 95°C for 9 min, 35 cycles of 95°C for 45 sec; 58-59°C for 45 sec; 72°C for 1 min; the final step at 72°C was extended for 7 min. The amplification products were subjected to agarose gel electrophoresis and stained with Syber Green (Invitrogen). PCR products were then purified (Quiagen) and sequenced.

Primers design and Real-Time PCR

Primer 3 program (http://frodo.wi.mit.edu/cgi-bin/primer/primer3.cgi/primer3_www.cgi) was used to design specific primers located on untranslated gene regions (UTR). The primers were characterized by a length of 20-24 nucleotides and a predicted melting temperature (Tm) of 59-61 °C (Table 1).

Transcript PIP levels were analyzed by Real Time PCR using the MJ Opticon 2 system (Bio-Rad) and SYBR Green JumpStart *Taq* ReadyMix (Sigma). Reactions were done in 20 μl final volumes containing 0.5 μM of each primer, 2 μl of cDNA appropriate dilution and 10 μl of 2X SYBR Green JumpStart Taq ReadyMix (Sigma; containing 100mM KCl, 20mM Tris-HCl, pH 8.3, 0.4 mM dNTPs, 0.05 U/μl Taq DNA polymerase, 7 mM $MgCl_2$, JumpStar Taq antibody, 2x internal reference dye and SYBR Green I). Aliquots from the same cDNA sample were used with all primer sets in each experiment.

The PCR cycling program consisted of one cycle of 2 min at 94°C, followed by 45 cycles of 94°C for 15 sec and 60°C for 1 min, with a final melt gradient starting from 50°C and heating to 95°C at a rate of 0.5°C s^{-1}. The efficiency of the primer set was evaluated by performing standard curve with five dilutions of cDNA and similar values were obtained.

The relative transcript abundance was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) with ubiquitin as a housekeeping gene; two other housekeeping genes were tested: actin and tubilin but the transcript levels of ubiquitin were found to vary least among different tissues. Ubiquitin has also previously been determined to have a stable transcription through development stages and over different tissues (Brunner et al. 2004).

Plasmid construction, *in vitro* cRNA synthesis and translation

The cDNAs encoding poplar PoptrPIP2s and *PoptrPIP1.1* were epitope-tagged at the C terminus with the c-Myc sequence, and tagged constructs were subcloned into the oocyte expression vector pT7TS. The epitope-tagged constructs consisting of each full-length aquaporin plus ten amino acids of the human c-Myc epitope (EQKLISEEDL at the carboxy-terminal end).

Tagged *P. trichocarpa* PIP cDNAs were first amplified by PCR using primers containing additional restriction enzyme recognition sites for *Xho*I and *Spe*I (Table 2): antisense primers were designed to incorporate the c-Myc tag in frame with the PIP protein followed by a stop codon and a *Spe*I restriction enzyme. Sense primers were designed to incorporate a unique restriction site (*Xho*I) and to anneal the coding region of each gene. .

The same conditions as described above were used in amplification. The amplified DNA fragments were digested, the inserts cloned in pT7TS vector using T4 ligase (Fermentas) and transformed into *Escherichia coli* HB101 Competent Cells (Promega). The transformed colonies were then purified with a Plasmid Midi Kit (Qiagen) and, to ensure proper PCR amplification, the cDNA products of the clones were sequenced. After linearization of the plasmid with *Bam*HI, capped complementary RNAs (cRNAs) were synthesized in vitro using T7 mMESSAGE mMACHINE Kit (Ambion Austin,TX) following the manufacturer's instructions, yielding PIP2 protein-encoding cRNAs.

Osmotic water permeability assay

Xenopus laevis oocytes were isolated and defolliculated as described by Hill (Hill et al. 2005). To determine an optimum mass of aliquot of PIPs cRNA to be injected to oocytes, we conducted an assay for different mass of *PIP2.3* cRNA injection: 2, 5, 10, 20 and 40 ng (see results). Based on these findings we used 20 ng aliquot for all remaining PIPs' cRNA or the corresponding volume of water. *Xenopus laevis* oocytes in stage V and VI were used for all injections. Injected oocytes were incubated in modified Barth's saline (MBS; 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO$_3$, 0.3 mM Ca(NO$_3$)$_2$, 0.41 mM CaCl$_2$, 0.82 mM MgSO$_4$, 15 mM HEPES), 10 µg ml^{-1} sodium penicillin and 10 µg ml^{-1} streptomycin sulfate, pH 7.4 at 18°C for

three days. Swelling tests were performed by moving oocytes to 50% MBS solutions. Images of swelling oocytes were acquired every 10 s for a period of 3 min using an Olympus dissecting microscope and captured with a Scion CFW-1312C CCD camera. Images were analyzed with ImageJ software (wsr@nih.gov). Two to three hundred oocytes were injected with each PIP cRNA or with the same volume of water (control) with total number of injections exceeding 2500. Healthy oocytes, eight to twelve, were selected for different treatments and ion combinations with more than 500 swelling assays performed.

To study the effect of heavy metals on oocyte swelling, some of the oocytes were incubated for 2 min in MBS containing either 1mM $NiCl_2$ or 1 mM Na_2WO_4 or $CuCl_2$ and then assayed as in 50% MBS devoid of metals. In addition, an assay was performed to test the effects of four copper concentrations (0.050, 0.250, 1 and 10mM of $CuCl_2$) on the P_f of oocytes injected with *PIP2.3*. To determine mercury sensitivity, oocytes were pre-treated for 30 min in MBS containing 100μM $HgCl_2$. We have also tested the effect of acidification on PIP2 encoded proteins' activity by incubation of oocytes in MBS pH 6.5 for 5 min prior to permeability (P_f) assay. The P_f of oocytes was calculated from the slope of the time course of change in oocyte volume following transfer to 50% MBS (Johansson et al. 1998), in these calculations oocytes were assumed to have and maintain spherical shape.

Isolation of oocyte crude lysate and immunoblotting

A crude preparation was made by homogenizing 40 oocytes (injected with cRNA encoding for poplar tagged proteins or injected with cRNA encoding for European eel *Anguilla anguilla* untagged aquaporin) in 400μl MBS plus protease inhibitors (Roche EDTA free complete mini tablets) by hand using a pestle. High density material was removed by

centrifugation at 500x *g* for 5 minutes. The supernatant was transferred to a clean tube and the

total protein level was assayed with Coomasie-Plus reagent (Pierce, Rockford, IL) with

spectrophotometric measurements made on a Biophotometer (Eppendorf, Westbury, NY). Equal

amounts of protein from each sample were denatured at 22 °C for 15 minutes in 4x sample buffer

and run on SDS-PAGE using 8-16% precast Long-life gels (NuSep Austell, GA) and then

electrotransferred to Immun-Blot polyvinylidene difluoride membrane (Bio-Rad Laboratories,

Hercules, CA) at 40V for 90 minutes. The membrane was blocked overnight in a TBS + 0.1%

v/v Tween-20 solution containing 5% skim milk powder. Monoclonal antibodies to the *c-Myc*

epitope 9E10 (Upstate-Millipore, Billerica, Massachusetts) were incubated at 1:500 for a

minimum of 3 hours at room temperature, washed, followed by 1:5000 horse-radish peroxidase

coupled anti-mouse secondary antibody (GE Life Sciences, Piscataway, NJ) and detected by

ECL (GE Life Sciences). Blots were exposed to standard X-ray film for various times.

Results

**Identification of the PIP2 family aquaporins in *P. trichocarpa* and phylogenetic analysis of
the deduced PIP proteins**

     Search of the *P. trichocarpa* genome in JGI database returned a total of 23 sequences

annotated as possible plasma membrane intrinsic proteins (PIPs). Four of them were removed

from further study; three (gw1.VI.1671.1, gw1.V.1886.1, eugene3.111150001) contained an

incomplete coding region (CDS) and one of them (fgenesh4_pg.c.lg_VI0336) shows a low

identity with few known PIP sequences (tblastx analysis). Also, the eugene.00090399 sequence

was not considered in this research because it has no EST support and was similar to another

EST supported sequence (*PoptrXIP1.1* for name see Table 1). For the additional sequences, to avoid the high homology among the coding regions of the PIP gene family, specific primers were designed on untranslated regions, the exceptions to this were *PoptrXIP1.2* and *PoptrXIP1.3* where only the coding region sequence was available and *PoptrPIP1.3* where primers designed on UTR region did not work.

PCR analysis was performed using wood cDNA as a template and for two sequences there was no amplification despite using two or three different primer pairs, suggesting that these are pseudo genes or they are not expressed in vegetative tissue (fgenesh4_pg.C_scaffold_57000043, eugene3.0009039). In all other cases aquaporin primers gave only one band of expected (bp) size (Table 1), the products of amplifications were confirmed by sequence analysis and in total 16 sequences were obtained.

Based on the alignment of poplar sequences with 27 other known plant plasma membrane intrinsic proteins, a neighbor-joining tree was constructed to determine classification of the deduced proteins. The 27 known sequences used for this analysis were selected from the model *Arabidopsis thaliana* plant and from *Populus tremula x tremuloides* (seven annotated PIP sequences) and from *Populus euphratica* (one annotated sequence). As shown in Fig. 1, aquaporin sequences of *P. trichocarpa* were distributed into three groups: PIP1, PIP2 and XIP subfamilies. These clusters were also obtained with analysis performed using a different algorithm such as UPGMA (data not shown). Eight genes were identified as putative PIP2 family genes, five genes were predicted to encode PIP1 subfamily, and the other three (*PoptrXIP1.1* and *PoptrXIP1.2*, *PoptrXIP1.3*), although deposited on the poplar genome as plasma membrane intrinsic proteins (PIP), appear to belong to a separate group. To clarify this distribution, an alignment of poplar PIPs was performed to compare the amino and carboxy-terminal extension

using the CLUSTALW program. All PoptrPIP1s showed a longer amino-terminal length and a shorter carboxy-terminal end compared with the PIP2 subfamily proteins. The same amino acid terminal length was found for XPI proteins (except for *PoptrXIP1.1*, which showed a longer carboxy-terminal length), even though these proteins had a weak overall sequence similarity to poplar PIP1s (about 25-30% amino acid identity) (data not shown). The proposed nomenclature for these proteins and their corresponding cDNA has been established according to the results of multiple alignments with PIP genes from *P. tremula* x *tremuloides* with respect to current nomenclature (Johanson et al. 2001).

Seven PoptrPIP2 putative proteins have highly similar aquaporin orthologous isolated from *P. tremula* x *tremuloides*: PoptrPIP2.2 has 98% amino acid identity with PttPIP2.2 (AJ849325), and PoptrPIP2.1 has 96% with PttPIP2.1 (CAH60720). PoptrPIP2.4 shows 99% similarity with PttPIP2.4 (CAH60723) and PoptrPIP2.3 is most similar to PttPIP2.3 (AJ849326) (94% identity). PoptrPIP2.5 has 96% similarity with PttPIP2.5 (AJ849328). Interestingly, most of the different amino acid residues between these protein sequences are located in loop C, the extracellular loop joining the transmembrane domains 3 and 4 (data not shown). Similarly, PttPIP1 proteins have an equivalent protein in the PoptrPIP1 family (e.g., PoptrPIP1.1 and PoptrPIP1.2 show 95% and 99% respectively with PttPIP1.1 (AJ849323) and PttPIP1.2 (AJ849322)).

Tissues-specific expression of PIP genes

The expression of the PIPs and XIP subfamilies of putative genes identified above were tested in different vegetative tissues including roots, wood, bark (including phloem) and leaves. The accumulation of aquaporin transcripts was tested by Real Time PCR using primers designed

for the gene specific untranslated sequence. Expression of aquaporins was related to the expression of the ubiquitin gene and the value obtained from *PoptrPIP2.1* in roots was set to 1.0 for comparison between genes and tissues in PIP2 subfamily and *PoptrPIP1.4* expressed in roots was set to be 1.0 for comparison among the members of PIP- XIP subfamily. All putative genes from the PIP2 family were expressed in each tissue analyzed but significant differences were present at the level of expression (Fig. 2): *PoptrPIP2.2* showed high transcript levels in all tissues. *PoptrPIP2.5*, *PoptrPIP2.6* and *PoptrPIP2.8* were abundant in leaves. *PoptrPIP2.7* is mostly expressed in root. Transcripts from *PoptrPIP2.4* displayed moderate accumulation while expression of *PoptrPIP2.1* was detected to low levels in all tissues (Fig. 2). All putative genes from the PIP1 family were expressed in vegetative tissues; *PoptrPIP1.1* and *PoptrPIP1.3* genes had the highest rates of expression, the *PopPIP1.3* transcript level in leaves was 2 times higher than in bark and about 5 times higher than in wood or roots. *PoptrPIP1.2* and *PoptrPIP1.5* showed moderate transcript accumulations in all tissues (Fig. 3). The expression of *PoptrXIP1.1 and PoptrPIP1.2* could be detected at very low levels in the root or wood, whereas *PoptrXIP1.3* was expressed in the leaf and bark.

Water channel activities of poplar PIP2 aquaporins and response to different treatments

The amount of PIPs cRNA to use in the assay was established after injection of 2, 5, 10, 20 and 40 ng of *PoptrPIP2.3* cRNA. A significant increase in P_f value was seen between 2 and 20 ng of aliquot, however, a further increase to 40ng did not differ from that of 20 ng injections (Fig. 4). Thus, 20 ng cRNA was used for all future assays.

During hypotonic treatment, control oocytes (water-injected) swelled slowly compared to all PIP2 expressing oocytes, whose osmotic water permeability was at times 5x higher than

controls (Fig 5). The presence of the c-Myc epitope tag attached to the C terminus end of each gene allowed us to test for the presence of aquaporin protein in oocytes. Western blot analysis experiment was carried out using *Xenopus* oocyte*s* microinjected with 20 ng of each cRNA and all myc-PIP2s were detected in oocyte crude fractions with the c-Myc antibody. All proteins were found to be present in the preparations. A lysate prepared from uninjected control oocytes confirmed that the anti Myc epitope tag antibody is specific to the expressed AQPs. Moreover, as a positive control, a lysate from *Xenopus* oocytes expressing an untagged aquaporin from the European eel *Anguilla Anguilla* was loaded and the results showed that no other protein is detected by the anti-c-Myc antibody (Fig.6).

The water channel activity was also tested for one of PIP1 gene subfamily. For this purpose, *PoptrPIP1.1* was chosen for its consistent expression in all tissues analyzed and for its primers specificity. The expression of *PoptrPIP1.1* proteins in *Xenopus laevis* did not show any significant differences in P_f when compared water injected oocytes (Fig. 5).

Assessment of heavy metals ($HgCl_2$, $CuCl_2$, $NiCl_2$, and Na_2WO_4) gating potential showed a range of responses, from gating ($HgCl_2$), to no response ($NiCl_2$ and Na_2WO_4), to stimulation of water membrane permeability ($CuCl_2$) (Fig. 7). The reduction in P_f caused by $HgCl_2$ ions was variable and ranged from no effect on PoptrPIP2.7 and PoptrPIP2.8, to a modest effect on PoptrPIP2.1, PoptrPIP2.6 and PoptrPIP2.5 (20-30%), to a more significant reduction in P_f, ranging from 44 to 67%, in PoptrPIP2.2, PoptrPIP2.4 and PoptrPIP2.3. As mentioned above, incubation of oocytes in 1mM $NiCl_2$ or with 1 mM Na_2WO_4 had no significant effect on the mRNA-dependent P_f. Surprisingly, incubation of all (except PoptrPIP2.1) tested PIP2s expressing oocytes in 1mM $CuCl_2$ showed an increase in water permeability by more than 50% (Fig 7).

A test of the effect of copper-concentration on PoptrPIP2.3 membrane permeability was undertaken to investigate the surprising enhancement of membrane P_f expressing PoptrPIP2s after exposure to copper ions. Four different concentrations: 0.050, 0.250, 1 and 10mM of $CuCl_2$ were used in the assay. P_f rose by a modest ~20% after exposure to 0.050 and 0.250 mM of $CuCl_2$. However, a significant increase in membrane permeability (~80%) was induced by concentrations above 1mMol of $CuCl_2$ (Fig. 8).

The response to acidification was tested by bathing oocytes in pH 6.5 buffer for 5 minutes prior to test. The results showed no significant effect on swelling kinetics of aquaporin-expressing oocytes suggesting that external pH had no effect on behavior of PIP2 proteins in oocytes membranes (Fig 7). The membrane permeability of the control water-injected oocytes used in each experiment was not affected by treatments that varied ions and pH (Fig. 7).

Discussion

Thirteen out of sixteen plasma membrane intrinsic proteins, annotated in the poplar genome database, can be separated into two groups classified as PIP1 and PIP2, similar to other plant PIP families. In addition three genes should be reclassified to a separate subfamily (XIP uncategorized intrinsic protein) already identified by Danielson and Johanson (2008). In presented work we describe these genes as PoptrXIP1.1, PoptrXIP1.2 and poptrXIP1.3. This uncategorized subfamily is present in many other plant species but very little information on the function, sub cellular location and expression pattern is available. As earlier reported these proteins had amino acid variations at the first NPA box and the ar/R filter suggesting that they are not functional water channels (Danielson and Johanson 2008).

Within the PIP2 subfamily, whenever there was a described gene from other *Populus* species, there was an orthologous gene in *P. trichocarpa* with 94 to 99% sequence identity. In fact, amino-acid sequence analysis within each pair of genes showed only minimal variation with less than four significant substitutions, mostly located in the extracellular loop C. All tested putative PIP2 genes were expressed in all vegetative plant tissues, although there were significant differences in the level of expression. The Poplar PIP1 family exhibited differences in transcript abundance among its members; and *PoptrXIP1.1 and PoptrXIP1.2* were not expressed in leaf and bark, whereas *PoptrXIP1.3* was detected only in vegetative tissue. The tissue-specific expression profile of some genes could suggest a different role in water uptake and transport in a particular area.

The functional activity of PIP1 subfamily was investigated only for PoptrPIP1.1, since proteins from this family usually exhibit little or no water channel activity when expressed in oocytes, although PIP1 proteins have been shown to interact with PIP2 isoforms to increase the membrane permeability (Fetter et al. 2004; Temmei et al. 2005).

On the other hand, PIP2 protein subfamily is considered to be composed of functional water channels. However, this was called into question by a recent report that PttPIP2.1 and PttPIP2.2 proteins, although expressed in plant, did not show transport capacity in oocytes (Marjanovic et al. 2005). The analysis presented here shows that all PIP2 proteins functioned as water channels when expressed in oocytes whereas PoptrPIP1.1 has no water activity in this system. Interestingly, Marjanovic et al. (2005) reported that the equivalent PttPIP2.2 PoptrPIP2.2 has no capacity to transport water. We found that *PoptrPIP2.2* is the most expressed gene and definitely functions as a water channel. The discrepancy might be the result of a lack of expression of *PttPIP2.1* and *PttPIP2.2* genes in oocytes in the former study (Marjanovic et al.

2005), since the authors did not confirm their expression in oocytes. Alternatively, the reported

lack of function might be the result of differences in amino-acid sequence between *P.*

trichocarpa and *P. tremuloides* genes. The only significant difference that we have found was

located in the third loop of both proteins. Similar substitutions were found in other equivalent

PoptrPIPs and PttPIPs genes and there was no significant change in their respective proteins

water channel activity. However, irrespective of the cause of these discrepancies, we

demonstrate that all PIP2 proteins from *P. trichocarpa* tested in this study function as water

channels.

Studies *in vivo* of aquaporin involvement in physiological functions often require the

ability to manipulate their activity. Gating by the use of heavy metals is the most widely used

technique to reduce membrane hydraulic conductivity, with mercury functioning as the most

pronounced inhibitor agent (Barone et al. 1997; Beaudette et al. 2007; Hukin et al. 2002). Indeed

in this study, it was the only metal that was able to gate the transport capacity of all PoptrPIP2,

except for PoptrPIP2.7 and PoptrPIP2.8 proteins. The mercury inhibitor capacity was in the

range of 30 to 50%, suggesting that mercury could potentially be carefully used in physiological

experiments to block water channels in *P. trichocarpa,* even if it is not possible to exclude $HgCl_2$

effects on plant transport systems. Other metals (tungsten and nickel) showed no influence on the

water transport capacity of all PoptrPIP2 proteins, despite reports that these treatments could

block aquaporin transport capacity in eel AQP3 (MacIver et al. In press) and human aquaporins

(Nemeth-Cahalan and Hall 2000; Yasui et al. 1999; Zelenina et al. 2003).

We tested the effect of altering external pH on the activity of the poplar aquaporins based

on the effect on the AQP3 class of mammalian aquaporins (MacIver et al. In press; MacIver et

al. 2008; Zeuthen and Klaerke 1999) and found no effect. We note that a histidine residue

located on loop D (intracellular side) of AtPIP2.2 was identified to be the major pH-sensing site (Tournaire-Roux et al. 2003). This histidine appears to be conserved in all PIP aquaporins identified to date. Our experiments were designed to affect only the external pH with insufficient time allowed to alter the internal pH. We would expect the poplar aquaporins to respond in a similar fashion to AtPIP2.2 when oocyte intracellular pH is reduced, but have not yet tested this hypothesis.

Interestingly, the presence of copper ions had a stimulating effect on oocytes P_f expressing all PoptrPIP2 proteins except for PoptrPIP2.1. This effect was responsible for as much as 50% increase in membrane permeability. In this study, we did not investigate the biophysical cause of this effect. It comes as a surprise that copper forms a part of the heavy metal group with silver and gold, which show strong inhibition of plant aquaporins in other studies (Niemietz and Tyerman 2002). The effect of copper on aquaporins could be a useful tool in the research of plant relations, particularly in studies where fast stimulation of aquaporin function is of interest. Of course, one has to remember that copper in high concentrations might show non-specific toxicity interacting with respiration pathways, and thus its use might have other physiological implications.

Conclusions

All eight PoptrPIP2 putative genes found in the *P. trichocarpa* genome are expressed in vegetative tissues. In all cases expressed proteins function as water channels based on their activity in oocytes. Mercury ions showed a modest inhibitor effect for six out of eight tested proteins, including the highly expressed PoptrPIP2.2 protein. Thus, mercury can be used as non-specific blocker for aquaporin activity in *P. trichocarpa*. Copper ions stimulated protein water

channel activity, suggesting its potential role in future physiological experiments. External

application of low pH, nickel and tungsten ions had no effect on aquaporin activity.

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Table 1: Gene name, JGI source and sequences of primers used for quantitative real time PCR (Q-RT-PCR).

Gene name	JGI source	Forward primer	Reverse primer	Product size (bp)	Primers Location
PoptrPIP2.1	estExt_fgenesh4_pg.C_LG_IX0306	5'-TCGGATTATGATGGACCTTTC	5'-ATGTGGTTGAGAAGGGAACG-3'	167	3'-UTR
PoptrPIP2.2	grail3.0045020302	5'-CCGCCAACTAAAGAGGAAAA-3'	5'-TGGGCAAAAGAAGAAAGGTC-3'	160	3'-UTR
PoptrPIP2.3	estExt_Genewise1_v1.C_LG_XVI2799	5'-GTGAGCTTGGGCACTTGTTT-3'	5'-CGTGAATTTCCTTCCCTGAC-3'	200	3'-UTR
PoptrPIP2.4	eugene3.00102165	5'-CTTTGAAAAGGGGAGGCTTT-3'	5'-AGTGGTGCATGCAGCTAAAA-3'	184	3'-UTR
PoptrPIP2.5	estEXT_FGENESH4_PG.C_280159	5'-TGGTGTAACGCAAAGATTCAAG-3'	5'-GAAGCAGCACTACCTGAGACAA-3'	165	3'-UTR
PoptrPIP2.6	eugene3.00080337	5'-TCTTCGAAAAGGGAGGCTTT-3'	5'-GCAGGAGAAATGTGATGATGAA-3'	185	3'-UTR
PoptrPIP2.7	estExt_fgenesh4_pg.C_LG_IX1411	5'-CCTTGTGCAATTGGGAAAAC-3'	5'-GACATAACATGACCCAACTGAGA-3'	159	3'-UTR
PoptrPIP2.8	estExt_fgenesh4_pm.C_570016	5'-CCTTGCGCTCTAAGAACCAC-3'	5'-TCTCTGTAGTCCTTCCCTCCA-3'	101	5'-UTR/ORF
PoptrPIP1.1	grail3.0049030302	5'-CAAGCCCAGTTTGTTCCATT-3'	5'-CAGCCAAACCCCTCAAACTA-3'	194	3'-UTR
PoptrPIP1.2	estExt_Genewise1_v1.C_LG_III0271	5'-TTCGCCCTTTCAAGAATCAC-3'	5'-AGGGAGGGAATGAAGCAAAT-3'	173	3'-UTR
PoptrPIP1.3	estExt_Genewise1_v1.C_LG_X1818	5'-GTGATGGAGGGCAAAGAAGA-3'	5'-ACAAGAAGGTGGCCATGAAC-3'	199	ORF
PoptrPIP1.4	estExt_fgenesh4_pm.C_LG_VI0336	5'-GTTTGGCTCTCAATTGTGTCTG-3'	5'-CCTTTCTGCAACACCTCACA-3'	151	3'-UTR
PoptrPIP1.5	estExt_fgenesh4_pm.C_LG_XVI0408	5'-CCCAATCAATGGATGTTTGA-3'	5'-GACGCAATTGAGAGCCAAAT3'	214	3'-UTR
PoptrXIP1.1	estExt_fgenesh4_pg.C_36020001	5'-AGAAGAGAACGATGGCTGGA-3'	5'-GATGCTCTCCATACCGTCAAA-3'	198	5'-UTR
PoptrXIP1.2	fgenesh4_pg.C_LG_IV001409	5'-TGGAGACAGGACAGGCTCTT-3'	5'-TTCCGACGATGATCAAAACA-3'	132	ORF
PoptrXIP1.3	estExt_fgenesh4_pg.C_LG_IX0381	5-'CTGGGCTTGGATGAACTTGT-3'	5'-AGGTTGCCATTACAAGGTTTG-3'	151	ORF
Ubiquitin	estExt_fgenesh4_pg.C_188960001	5'-CAGCTTGAAGATGGGAGGAC-3'	5'-CAATGGTGTCTGAGCTCTCG-3'	154	ORF

Table 2: Sequence of primers used for amplification of full-length cDNAs with epitope tags.

Primer code	Forward sequence	Reverse sequence
c-Myc/PIP2.1	5'-CCGCTCGAGGGAAAATGTCAAAGGACGTGATTGAGGAA-3'	5'-GGACTAGTCCCTATCACAGATCCTCTTCTGAGATGAGTTTTTGTTCATTTGTGGGGTGGCTACGGAA-3'
c-Myc/PIP2.2	5'-CCGCTCGAGGGAAAATGTCAAAGGAAGTGAGTGAAGTAGGG-3'	5'-GGACTAGTCCCTATCACAGATCCTCTTCTGAGATGAGTTTTTGTTCGTTGGCGGGGTTGCTACG-3'
c-Myc/PIP2.3	5'-CCGCTCGAGGGAAAATGGGCAAGGACATTGAAGTTGGAGGC-3'	5' -GGACTAGTCCCTATCACAGATCCTCTTCTGAGATGAGTTTTTGTTCTATGTTGGGAGAGCTCCGGAAGGAT-3'
c-Myc/PIP2.4	5'- CCGCTCGAGGGAAAATGGCGAAGGACATGGAAGTTGCAG-3'	5'-GGACTAGTCCCTATCACAGATCCTCTTCTGAGATGAGTTTTTGTTCAAACCGTTGGGCGCTCCTGAA-3'
c-Myc/PIP2.5	5'-CCGCTCGAGGGAAAATGGGCAAGGACATTGAAGTTGGA-3'	5'-GGACTAGTCCCTATCACAGATCCTCTTCTGAGATGAGTTTTTGTTCAATGTTGGAAGAGCTCCTGAAGGA-3'
c-Myc/PIP2.6	5'-CCGCTCGAGGGAGCATGGCAAAGGATACTGAAGTGGCAGAG-3'	5'-GGACTAGTCCCTATCACAGATCCTCTTCTGAGATGAGTTTTTGTTCGACATTCGGGTTGCTCCTGAA-3'
c-Myc/PIP2.7	5'-CCGCTCGAGGGAGTATGGCGAAGGATATTGAGGTTGC-3'	5'-GGACTAGTCCCTATCACAGATCCTCTTCTGAGATGAGTTTTTGTTCAGCATTGCTCCTGAAGGATCC-3
c-Myc/PIP2.8	5'-CCGCTCGAGGGAAAATGAGTACTGGAGGGAAGGACTACAGA-3'	5'-GGACTAGTCCCTATCACAGATCCTCTTCTGAGATGAGTTTTTGTTCGCTAGTAGGATTGCTGCGGAA-3'
c-Myc/PIP1.1	5'-CCGCTCGAGGGAAAATGGAGGGCAAAGAAGAAGATGTTAG-3'	5'-GGACTAGTCCCTATCACAGATCCTCTTCTGAGATGAGTTTTTGTTCCTTCTTGAAAGGAATGGCTCT-3'

Figures

Figure 1. Neighbor-joining tree of 44 PIP proteins based on the deduced amino acid sequences aligned by ClustalW. The program MEGA 4 was used for tree analyses. The black triangles and circles indicated, respectively, *Populus trichocarpa* PoptrPIP2 and PoptrPIP1 proteins. The sequence sources were: *Olea europaea*, OePIP2.1 (DQ202709), OePIP1.1 (DQ202708); *Arabidopsis thaliana*, AtPIP1.1 (P61837), AtPIP1.3 (AAP13421), AtPIP1.4 (Q39196), AtPIP1.2 (Q06611), AtPIP1.5 (Q8LAA6), AtPIP2.5 (Q9SV31), AtPIP2.3 (NP_181255), AtPIP2.4 (NP_200874.1), AtPIP2.2 (AAM63463), AtPIP2.1 (AAM65406), AtPIP2.6 (Q9ZV07), AtPIP2.8 (Q9ZVX8), AtPIP2.7 (P93004); *Fraxinus excelsior*, FePIP1.1 (AAT74898); *Populus tremula x tremuloides*, PttPIP2.5 (AJ849328), PttPIP2.4 (CAH60723), PttPIP2.1 (CAH60720), PttPIP2.2 (AJ849325), PttPIP2.3 (AJ849326), PttPIP1.1 (AJ849323), PttPIP1.2 (AJ849322); *Juglans regia*, JrPIP2.1 (AAO39007), JrPIP2.2 (AAO39008); *Populus euphratica*, PeupPIP2.a (AJ777168); *Prunus persica*, PpPIP1 (BAF62342.1); *Pyrus communis*, PcPIP2.1 (BAB40141).

Figure 2. Relative expression levels of the PIP2 subfamily genes in different vegetative tissues (leaf, root, wood and bark) of *Populus trichocarpa.* The transcript levels of each PIP2 gene were plotted as the relative expression fold of the PIP2.1 in root. The data are mean values and standard errors (bar) of two independent biological groups each group composed of two plants.

Figure 3. Relative expression levels of the PIP1 subfamily genes in different vegetative tissues (leaf, root, wood and bark) of *Populus trichocarpa.* The transcript levels of each PIP1 gene were plotted as the relative expression fold of the PIP1.4 in root. The data are mean values and standard errors (bar) of two independent biological groups each group composed of two plants.

Figure 4. Relationship between cRNA mass injected into oocytes and changes in membrane hydraulic permeability (P_f). This test was performed on PoptrPIP2.3 cRNA construct. Oocytes were injected with 2, 5, 10, 20 and 40 ng of cRNA, the maximum change of P_f was observed at ~20 ng of aliquot. The results are expressed as means of measurements from 8 to 12 oocytes. Vertical bars denote standard errors.

Figure 5. Water channel activity of poplar PIP2 and PoptrPIP1.1. Oocytes were injected with 20 ng of cRNAs enconding each protein or with the same volume of water (control) three days before the assay. P_f values are means and vertical bars denote standard errors of eight to twelve healthy oocytes. In all cases a significant increase of P_f was observed at $p<0.0001$.

Figure 6. Western blot of *C-myc* tagged poplar aquaporins. Crude lysates were prepared from approximately 40 whole *Xenopus* oocytes and 40μg protein was loaded on each lane after denaturing at 22 °C in 4x sample buffer for 15 minutes. The control lanes (last two lanes) are lysates prepared respectively from uninjected control oocytes and from *Xenopus* oocytes expressing an untagged aquaporin from the European eel *Anguilla*

Anguilla. There is no background signal and no other proteins are detected by the anti-*C-myc* antibody. On this blot the proteins predominate around the 50kDa mark which may mean that they are running as dimers, but it is also possible that denaturation is incomplete and an aberrant molecular weight ensues. The blots usefulness is in confirming that the proteins were expressed

Figure 7. Changes in oocytes P_f in response to presence of metal ions in external solution (mercury, copper, nickel, tungstate) and response to change of pH from 7 to 6.5. Significant difference is based on t-Student test between no-treatment and each treatment run separately where (*) - $p<0.05$, (**) - $p<0.01$ and (***) - $p<0.001$. The results are expressed as means of measurements from 8 to 12 oocytes. Vertical bars denote standard errors.

Figure 8. Effect of $CuCl_2$ on membrane hydraulic permeability (P_f). This test was performed on *PoptrPIP2.3* using 0.050, 0.250,1 and 10mM of $CuCl_2$. The maximum change of P_f was observed at 1 mM and 10 mM of copper. Pf values are means ± SD of eight to twelve healthy oocytes.
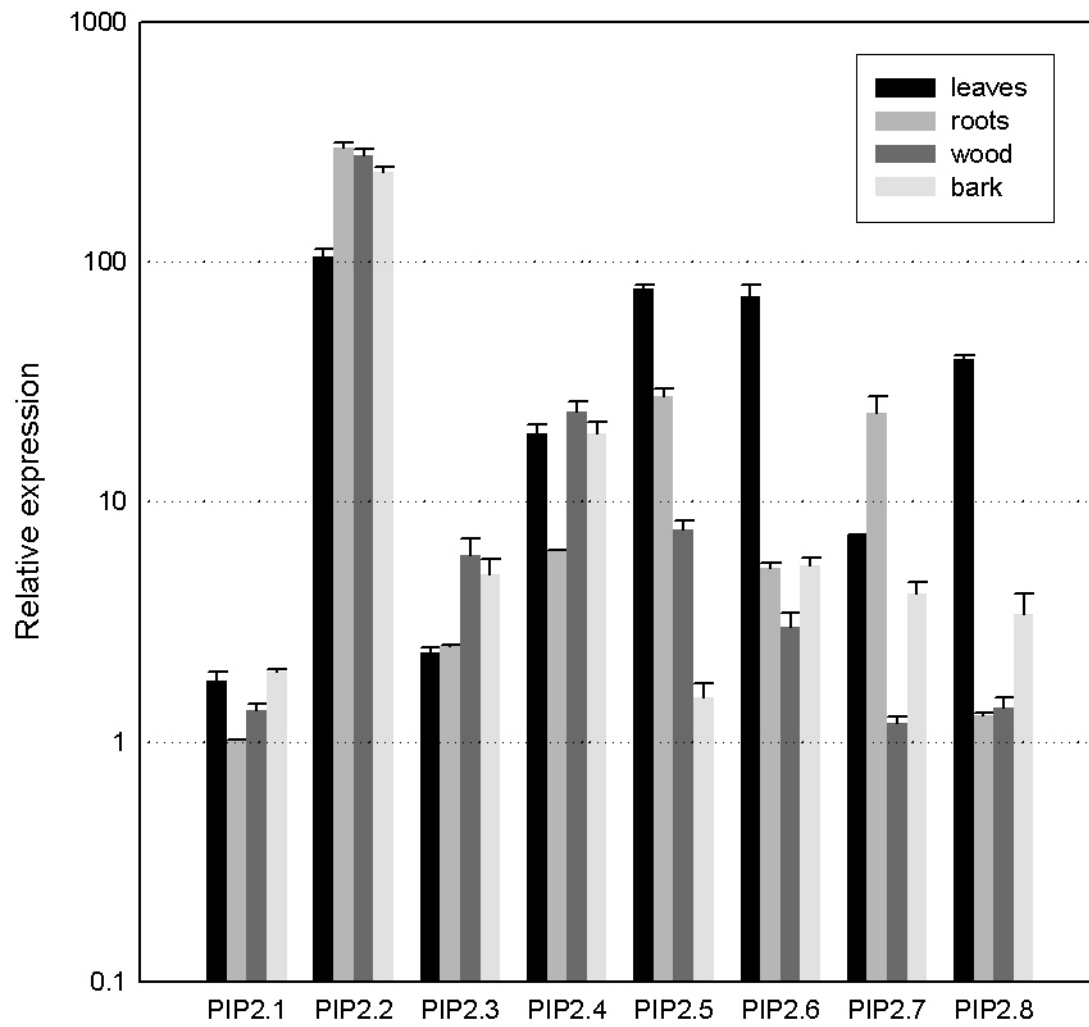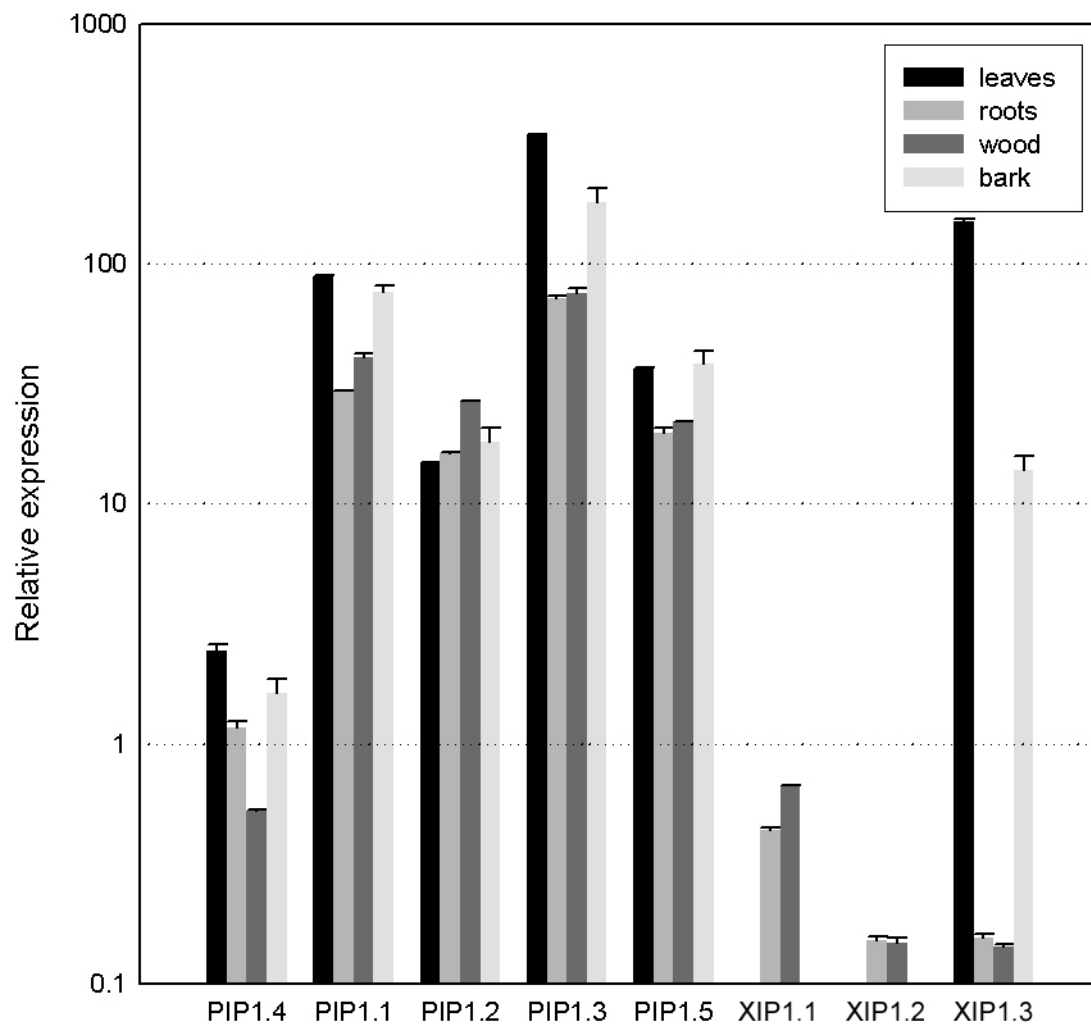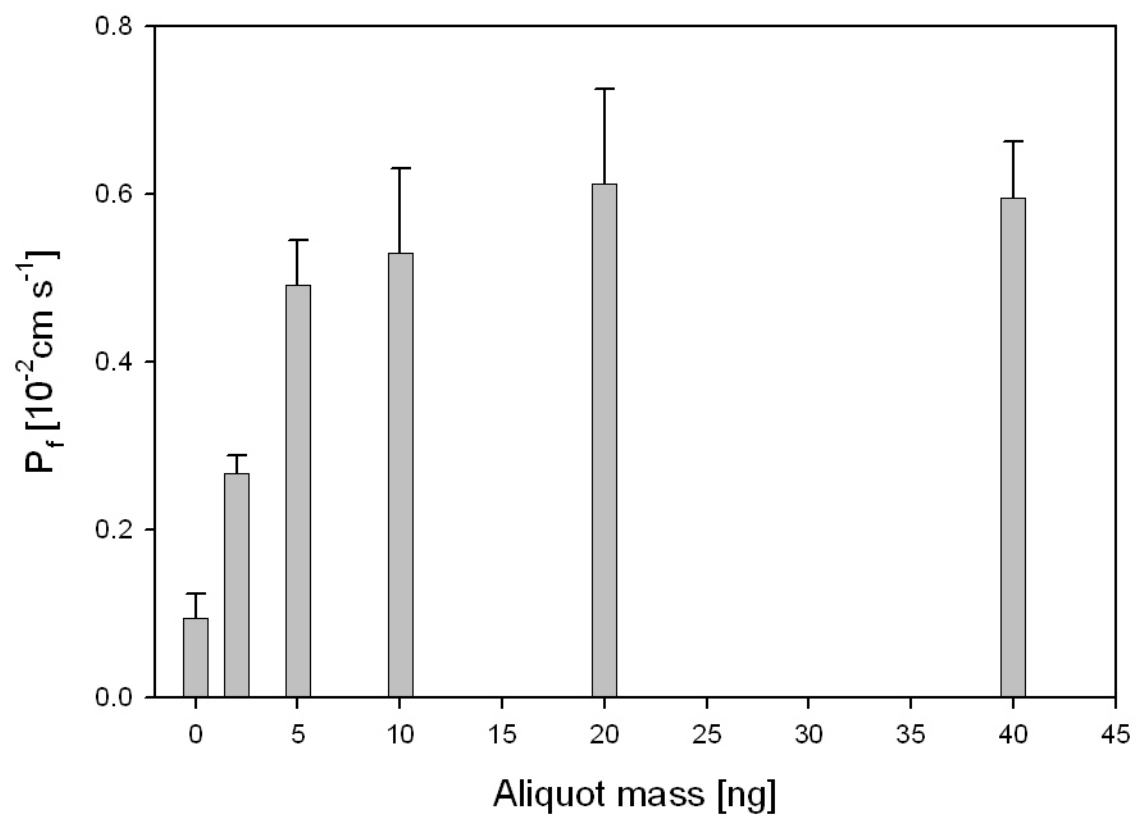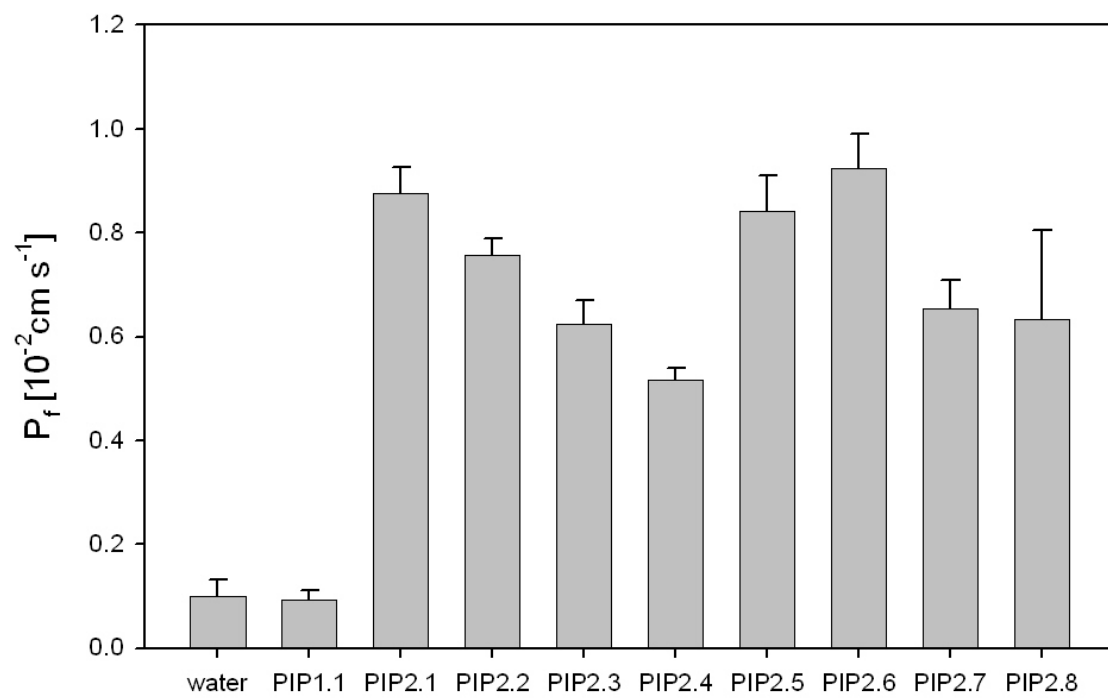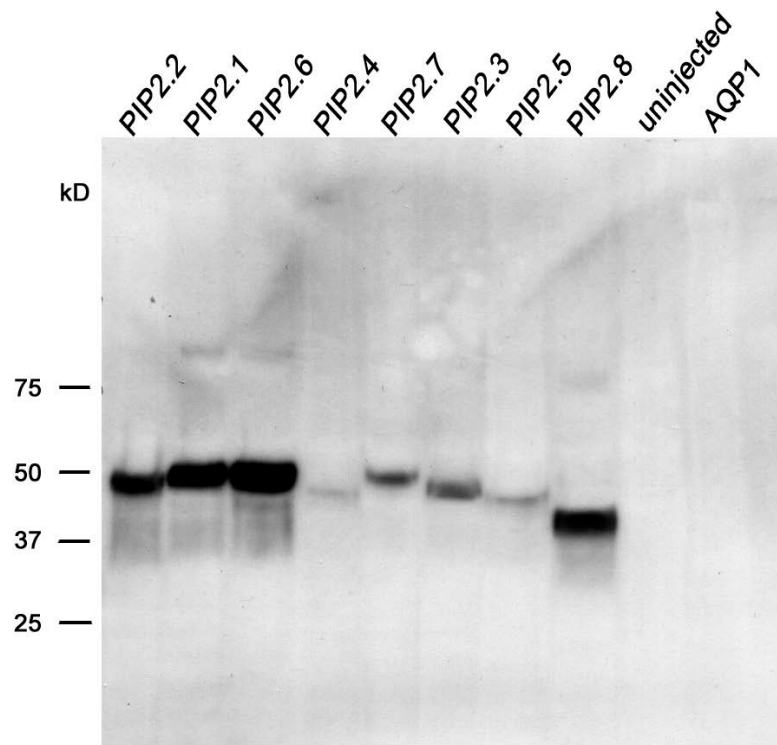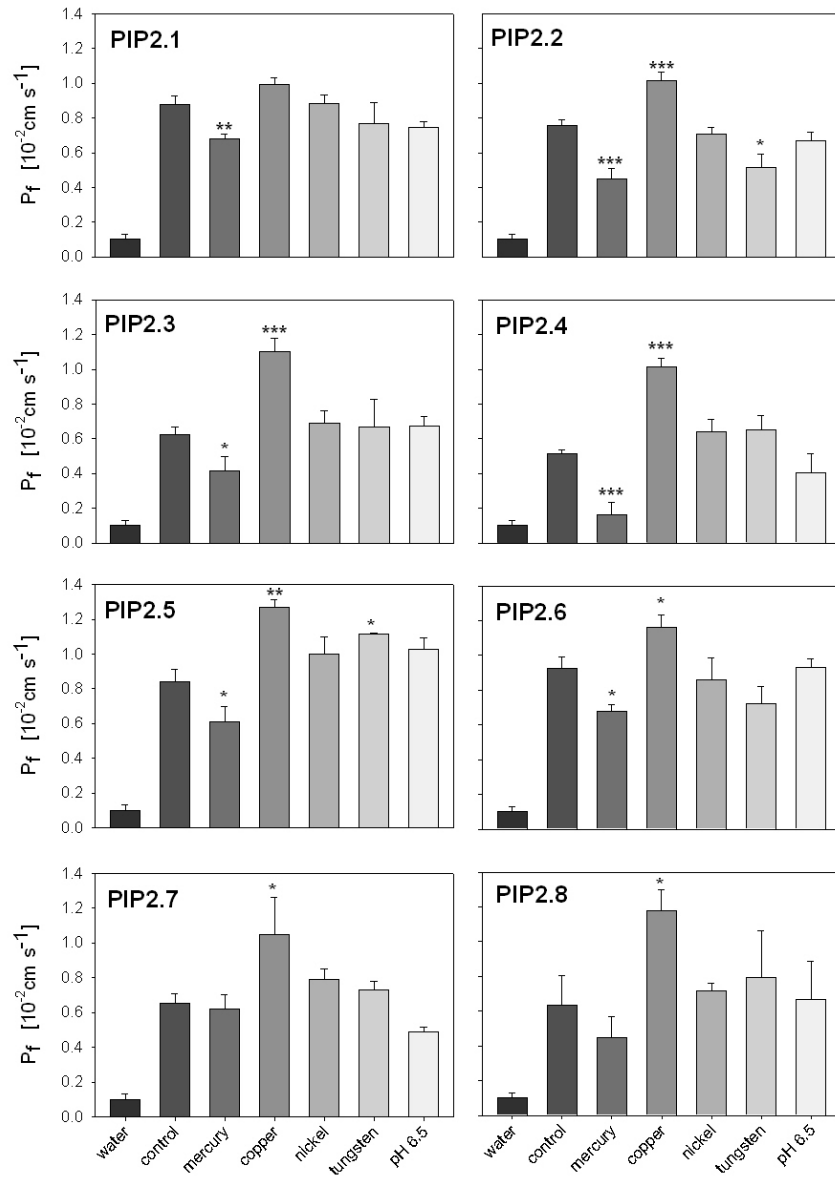
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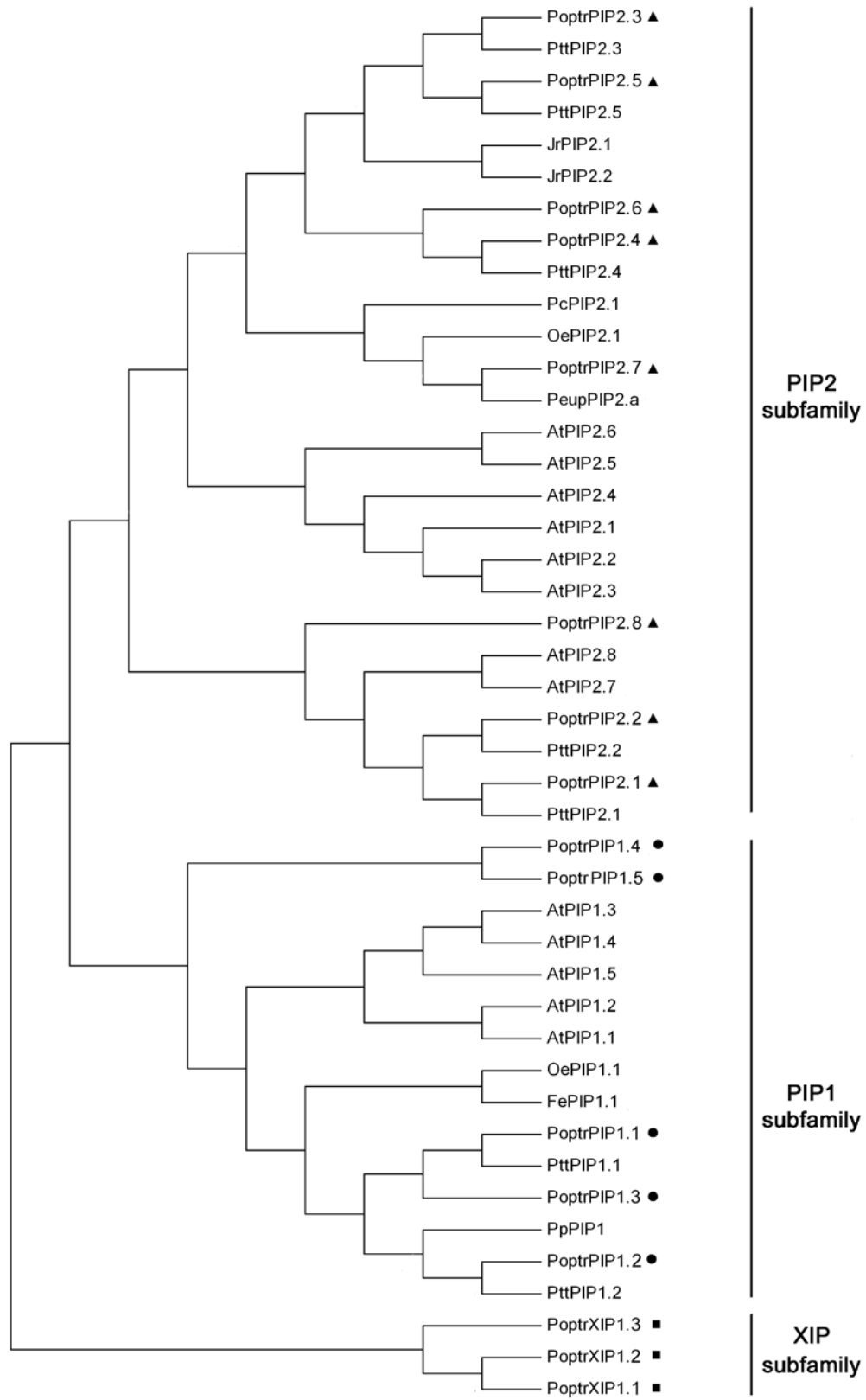
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