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Patterns of PIP gene expression in *Populus trichocarpa* during recovery from xylem embolism suggest a major role for the PIP1 aquaporin subfamily as moderators of refilling process

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ABSTRACT

Embolism and the refilling of xylem vessels are intrinsic to the ability of plants to handle the transport of water under tension. Although the formation of an embolized vessel is an abiotic process, refilling against the pressure gradient requires biological activity to provide both the energy and the water needed to restore xylem transport capacity. Here, we present an analysis of the dynamics of embolism and refilling in *Populus trichocarpa* and follow temporal dynamics of co-occurring changes in expression level of aquaporins. Under mesic conditions, we found that the percent loss of conductance (PLC) varied diurnally by as much as 20%, suggesting a continuous embolism/refilling cycle. An increase in water stress tilted the balance between the two processes and increased the PLC to as much as 80%. Subsequent re-watering resulted in the reversal of water stress and recovery of PLC to pre-stress levels. Stem parenchyma cells responded to drought stress with considerable up-regulation of the PIP1 subfamily of water channels but not the PIP2 subfamily. Even more significant was the finding that *PoptrPIP1.1* and *PoptrPIP1.3* genes were up-regulated in response to embolism, but not to water stress, and were down-regulated after embolism removal, suggesting a local ability of plants to sense an embolism presence.

1 *Key-words*: aquaporin; drought stress.

INTRODUCTION

Embolism in xylem vessels results in the loss of stem hydraulic conductance and subsequently loss in plant productivity (Tyree & Sperry 1989; Hölttä *et al.* 2009). It is commonly assumed that embolism is a result of severe drought stress (Tyree *et al.* 1994; Davis *et al.* 2002). However, growing evidence suggests that it can also be a daily cyclical phenomenon occurring even under conditions of low water stress (Zwieniecki & Holbrook 1998). This means that embolism has to be followed by vessel refilling and restoration of hydraulic capacity (McCully, Huang & Ling 1998; Domec *et al.* 2006; Lovisolo & Schubert 2006; Taneda & Sperry 2008). Although embolism formation is a purely physical process, related to the degree of tension in the water column and wood physicochemical properties (Tyree & Zimmermann 2002), vessel refilling requires physiological activity to provide both energy and water (Holbrook & Zwieniecki 1999; Tyree *et al.* 1999). Until recently, growing new vessels and/or generation of root pressure were considered the only options for regaining the lost transport capacity. These processes are either very slow or require the presence of a positive pressure in the plant. Therefore, we have to consider the ability of plants to refill vessels during active transpiration and on a much faster time scale (minutes to hours), when the bulk of a plants apoplastic water remains under tension (Holbrook & Zwieniecki 1999; Trifilo' *et al.* 2003). Despite significant scientific effort, our understanding of biophysical processes responsible for embolism refilling still remains elusive.

The existence of refilling under tension has been experimentally demonstrated using both *in vitro* and *in vivo* techniques (Salleo *et al.* 1996; Holbrook & Zwieniecki 1999; Tyree *et al.* 1999; Holbrook *et al.* 2001; Melcher *et al.* 2001) and interlinked with some basic aspects of physiology like sugar metabolism and phloem transport (Lampinen & Noponen 2003; Salleo *et al.* 2004). However, a comprehensive explanation of the process is still missing and is hotly debated. The common bases for these debates are the sources of energy and water needed to restore xylem transport activity (De Boer & Volkov 2003). It is parsimonious to assume that living cells can provide both. This assumption is supported by the inhibition of refilling in the case of either physical damage to phloem or metabolic inhibition of living cells in stems (Salleo *et al.* 2004; Zwieniecki *et al.* 2004). The aspects of cell biology that change during the process of refilling remain unresolved. Because the prevalent expectation is that xylem parenchyma cells supply water for refilling, or at least for part of it, we can assume that water channel proteins (PIP) are involved in the process. Studies on walnut (*Juglans regia*) showed that higher expression of

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two PIP2 genes (*JrPIP2.1* and *JrPIP2.2*) was observed in vessel-associated parenchyma cells at the same time that embolism refilling took place (Sakr *et al.* 2003). Further support for the involvement of water channels in dealing with adverse condition of water stress comes from observations that a reduction in the expression of PIPs in *Arabidopsis thaliana* plants impairs their ability to recover from water stress (Martre *et al.* 2002). However, no systematic research has yet been presented that includes measurements of the expression levels of the family of PIP proteins during the onset of embolism formation and recovery. In this work, we show the dynamics of expression levels of both PIP2 and PIP1 protein subfamilies in *Populus trichocarpa* wood during the onset of water stress and during the rapid recovery from stress involving xylem refilling.

P. trichocarpa is an obvious choice for studying gene-level responses because of the numerous genomic tools that are now available for this species. *P. trichocarpa* has already been established as a 'woody plant' model system (Brunner, Busov & Strauss 2004), and its entire genome has recently been sequenced (http://genome.jgi-psf.org/ Poptr1/Poptr1.download.html). Furthermore, poplar aquaporin genes were previously assayed for their expression in wood tissue and for their function as water channel proteins (Secchi *et al.* 2009). However, despite 'model plant' status, limited physiological data is available relating to water stress, embolism and refilling (Cochard, Ridolfi & Dreyer 1996; Harvey & Van Den Driessche 1997; Sparks & Black 1999). Studies on other poplar species suggest that trees from this genus are highly productive plants displaying fast growth when water availability is not a limiting factor (Rood, Braatne & Hughes 2003). Despite some diversity in water use efficiency and drought tolerance occurring between different species and clones, the genus is known to be drought-sensitive and vulnerable to water stress-induced embolism. Poplars are among the most vulnerable of measured woody species, cavitating massively even at very low water stress (~ −1 MPa) (Tyree *et al.* 1994; Cochard *et al.* 1996; Hacke & Sauter 1996; Harvey & Van Den Driessche 1997; Hukin *et al.* 2005). Based upon these physiological traits *P. trichocarpa* is likely to be a good model organism to study the physiology of embolism and refilling in addition to its status as a genetic 'model woody plant'. Here, we assess its vulnerability to cavitation in response to water stress. We then measure diurnal patterns of embolism formation and xylem refilling, with simultaneous measurements of changes in PIP gene expression during water stress and stress recovery.

MATERIALS AND METHODS

Plant materials and growth conditions

P. trichocarpa cuttings were rooted in aerated hydroponics containers (6.5 L) filled with modified Hoagland solution (pH 6.00–6.50; 795 *m*m KNO_3, 603 *m*m $Ca(NO_3)$ 270 *m*m $MgSO_4$ and 109 *m*m KH_2PO_4; micronutrients: 40.5 *m*m Fe(III)- ethylenediaminetetraacetic acid (EDTA), 20 *m*m H_3BO_4, 2 *m*m $MnSO_4$, 0.085 *m*m $ZnSO_4$, 0.15 *m*m $CuSO_4$ and 0.25 *m*m Na_2MoO_4) and located in a growth chamber (18/21°C, 10 h d night^{-1} periods, 60% humidity with a 500 *m*mol photons m^{-2} s^{-1} illumination). After 2 weeks, plants were transferred to 42 L boxes (12 plants per box) and the medium was replaced with low nitrate (5%) and otherwise half-strength Hoagland solution to promote root growth (pH 6.00–6.50; 39.75 *m*m KNO_3, 30.15 *m*m $Ca(NO_3)_2$, 135 *m*m $MgSO_4$ and 54.5 *m*m KH_2PO_4, 397.5 *m*m K_2SO_4, 301.5 $CaCl_2$; micronutrients: 20.25 *m*m Fe(III)-EDTA, 10 *m*m H_3BO_4, 1 *m*m $MnSO_4$, 0.0425 *m*m $ZnSO_4$, 0.075 *m*m $CuSO_4$ and 0.125 *m*m Na_2MoO_4). Six-week-old plants were then transferred into 1 gal pots filled with potting mix and were grown in a greenhouse for 3 months (December–February). Ambient conditions in the greenhouse depended somewhat on the external weather conditions, but the temperature was maintained in the range 17 to 29 °C and natural daylight was supplemented with light from metal halogen lamps (500–600 *m*mol photons m^{-2} s^{-1}) to maintain 12/12 h of light/night cycle.

Experimental design and water stress treatments

The experiment was designed to follow the diurnal dynamics of embolism formation and repair in stems. A total of 82 *P. trichocarpa* plants (of homogeneous size) were used in the water stress study, of these 30 plants were kept as controls. These plants were watered to field capacity daily around 0800 h. Water stress was imposed in succession on the remaining 52 plants by a reduction of irrigation. Depending on drought duration, two levels of water stress were achieved as confirmed by the stem water potential measurement: moderate stress with stem water potential averaging around −0.8 MPa and severe water stress with stem water potential averaging around −1.4 MPa. Twenty-two plants from the stressed treatment were used to follow diurnal pattern of embolism formation. The remaining 30 plants were used to study physiology during stress recovery after irrigation to field capacity the following day at 0900 h.

Diurnal variability of stem water potential and percent loss of conductance (PLC) were measured throughout the day. For control and water-stressed plants, the physiological measurements were performed from 0500 to 2100 h. Measurements on plants recovering from stress started as soon as 1 h after irrigation and continued until 2000 h, with one final measurement implemented the following day at 0900 h.

Measurements of stem water relations

Stem water potential was measured for each plant using mature leaves. Leaves were placed in a humidified plastic bag covered with aluminium foil for 15 min prior to excision and measurement. This treatment was applied to stop transpiration and allow time for water potential equilibration between leaf and the stem. Analysis of rehydration kinetics of poplar leaves suggested that 15 min was an ample time

length for equilibration to occur (Zwieniecki, Brodribb & Holbrook 2007). After excision, water potential was measured using Scholander-type pressure chamber (Soil Moisture Equipment Corp., Santa Barbara, CA, USA).

Following the determination of stem water potential, stem hydraulic conductivity was measured for each plant using a standard approach (Melcher, Zwieniecki & Holbrook 2003). In short, small sections of stems (~4 cm long) were cut under water directly from intact plants. Since plants were relatively young and stems flexible we were able to fully submerge stems in water during the cut to prevent embolisms caused by air entering into the cut vessels. The initial hydraulic conductance (k_i) of each stem segment was measured gravimetrically by determination of flow rate of filtered 10 mm KCl solution. A water source was located on a balance (Sartorius ± 0.1 mg) and connected to the stem by a plastic tube. The stem was submerged in a water bath with the water level being ~10 cm below the level of water on the balance. Such a small head of water was used to prevent the flooding of embolized conduits or the pushing of air out of open empty vessels. After a steady flow rate was reached (within just a few minutes), the tube connecting the stem to the balance was closed, and a bypass used to push water across the segment under ~2 bars of pressure for approximately 10 s to remove embolism. The chosen segment length was short enough to have the majority of vessels open in poplar stems (vessel length is usually >5 cm), thus making removal of embolism very easy and complete within few seconds (Melcher *et al.* 2003). Stem conductance was then re-measured to find maximum conductance (k_{max}). The *PLC* was calculated as:

$$PLC = 100*(k_{max} - k_i) \;\; k_{max}$$

Artificial pressure injection in stems

A separate group of 46 plants was used to determine recovery from artificially induced embolism. All plants were always maintained in a well-irrigated state (same as control plants). The injection treatment followed a simple procedure (Salleo *et al.* 1992, 1996, 2004; Sperry & Ikeda 1997). Firstly, stem water potential was measured on each tree prior to treatment (as described previously). Then an incision was made in the stem and small custom built pressure system was attached. The system consisted of a small diameter tube (0.98 mm) that was sealed to a incised stem using a custom build holder and allowed for delivery of pressure into the stem. We applied air pressure such that an equivalent of moderate stress ~ -1 MPa was reached (as the sum of applied pressure and plant balancing pressure). We pressurized stems for 60 s after which time the pressure was released and holder removed. Air injection was followed by determination of stem PLC over a 3 h period. Stem section assayed for PIP expression was located ~10 cm above where the chamber was attached. Since we used short stem segments located above the pressure application point we did not typically observe a period during which stems expelled a large quantity of gas. Open vessels degassed very fast for there was no bordered pit fields to slow the process, while location of the segment in distal direction from the injection removed the potential of pressurization of fibres and their prolonged degassing that might prohibit immediate conductivity measurements. Control plants were treated exactly the same way as injected plants including incision and chamber placement though no pressure was applied.

Plant material harvest and total RNA isolation

Wood samples for gene expression analysis (bark and phloem removed) were collected from the same plants that were used to determine the plant water status, stem hydraulic conductivity and response to artificially induced embolism. All samples were immediately frozen in liquid nitrogen and kept at -80°C. The diurnal expression pattern of PIP genes in control plants was monitored on samples collected from four periods (0800–1000 h; 1100–1300 h; 1400–1600 h; 1700–1900 h). Stressed plants and recovery plants were collected in two periods (0800–1000 h; 1100–1300 h). Samples were collected from plants immediately following the pressurization followed by additional samples between 0.5 and 0.75 h after injection and 2 h after injection.

We used a design with three biological replicates per collection time and treatment. Collected wood samples were grounded to a fine powder in liquid nitrogen and 100 mg of powder from each tree (two or three plants) was mixed to obtain one biological replicate. Each biological replicate was then analysed for expression a total of three times (technical replicates used to reduce variation introduced by small procedural differences). These three technical replicates were averaged for each biological replicate, providing a total of three biological replicates in the final statistical analysis. Total RNA was isolated according to the protocol of Chang (Chang, Puryear & Cairney 1993) and contaminant genomic DNA was removed from the samples by digestion with RNase-free DNase I (Fermentas, Vilnius, Lithuania), following the manufacturer's instructions. The concentration of RNA was quantified by measuring the absorbance at 260 nm and its integrity was checked on agarose gels.

cDNA synthesis and semi-quantitative real-time PCR

cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen) according to supplier's instruction, and using oligo-(dT)12–18 (Fermentas) as a primer.

The SYBR Green JumpStart *Taq* ReadyMix (Sigma, St Louis, MO, USA) was used to perform reactions with the MJ Opticon 2 system (Bio-Rad, Hercules, CA, USA); amplification conditions and primers design were described in detail in Secchi *et al.* (2009). The comparative method (Livak & Schmittgen 2001) was used to evaluate expression levels of the selected genes and the values were normalized relative to transcript levels of the ubiquitin gene. Transcript

levels were expressed relative to the average value at the time interval 0800–1000 h of control plants, which was set as 1.

Expression of poplar aquaporins in *Xenopus laevis* oocytes

The epitope-tagged constructs consisting of *Xho*I-*Spe*I full-length PIP2s and *PoptrPIP1.1* cDNA plus ten amino acids of the human c-Myc epitope (EQKLISEEDL at the carboxy-terminal end) were amplified by PCR using the primers listed in Secchi *et al.* (2009). The tagged constructs were inserted into the oocyte expression vector pT7TS and introduced into *Escherichia coli* HB101 Competent Cells (Promega, Madison, WI, USA) as described previously (Secchi *et al.* 2009). The constructs were linearized with *Bam*HI, and capped cRNAs were synthesized using the T7 mMESSAGE mMACHINE Kit (Ambion, Austin, TX, USA).

cRNA injection and osmotic water permeability assay

X. laevis oocytes were isolated and defolliculated as described by Hill (Hill *et al.* 2005). Approximately 30 nL distilled water (vehicle control) or 20 ng of cRNA were injected and the 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 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)], 10 *m*g mL^{-1} sodium penicillin and 10 *m*g mL^{-1} streptomycin sulfate, pH 7.4 at 18°C for 3 d. To measure the osmotic water permeability (P_f), oocytes were transferred to 50% MBS solutions and the increase in volume was observed by video microscopy. Determination of P_f followed procedure described previously (Secchi *et al.* 2009).

RESULTS

Diurnal trends in embolism and gene expression in control and stressed plants

P. trichocarpa proved to be very susceptible to cavitation. An embolism vulnerability curve obtained from measurements relating stem water potential of transpiring plants with PLC showed that a 50% loss of conductivity occurred at around −0.9 MPa (Fig. 1). Severe loss of conductivity (>80%) occurred around −1.25 MPa (a rather moderate level of stress in comparison with many woody species). This susceptibility to embolism was well reflected in an analysis of diurnal dynamics of PLC. Even well-watered plants (control) showed significant diurnal changes in PLC level despite a relatively constant stem water potential (Y_{stem} averaged −0.33 ± 0.074 MPa; Fig. 2). In the well watered plants a non-linear (quadratic) model was found to explain significantly more variation than a linear model of diurnal behavior of PLC [F(1,32) = 23.66 and $P < 0.0002$], suggesting that PLC did vary over the day. The control

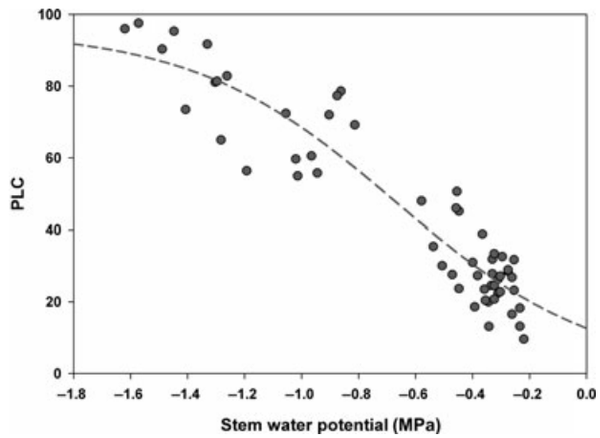
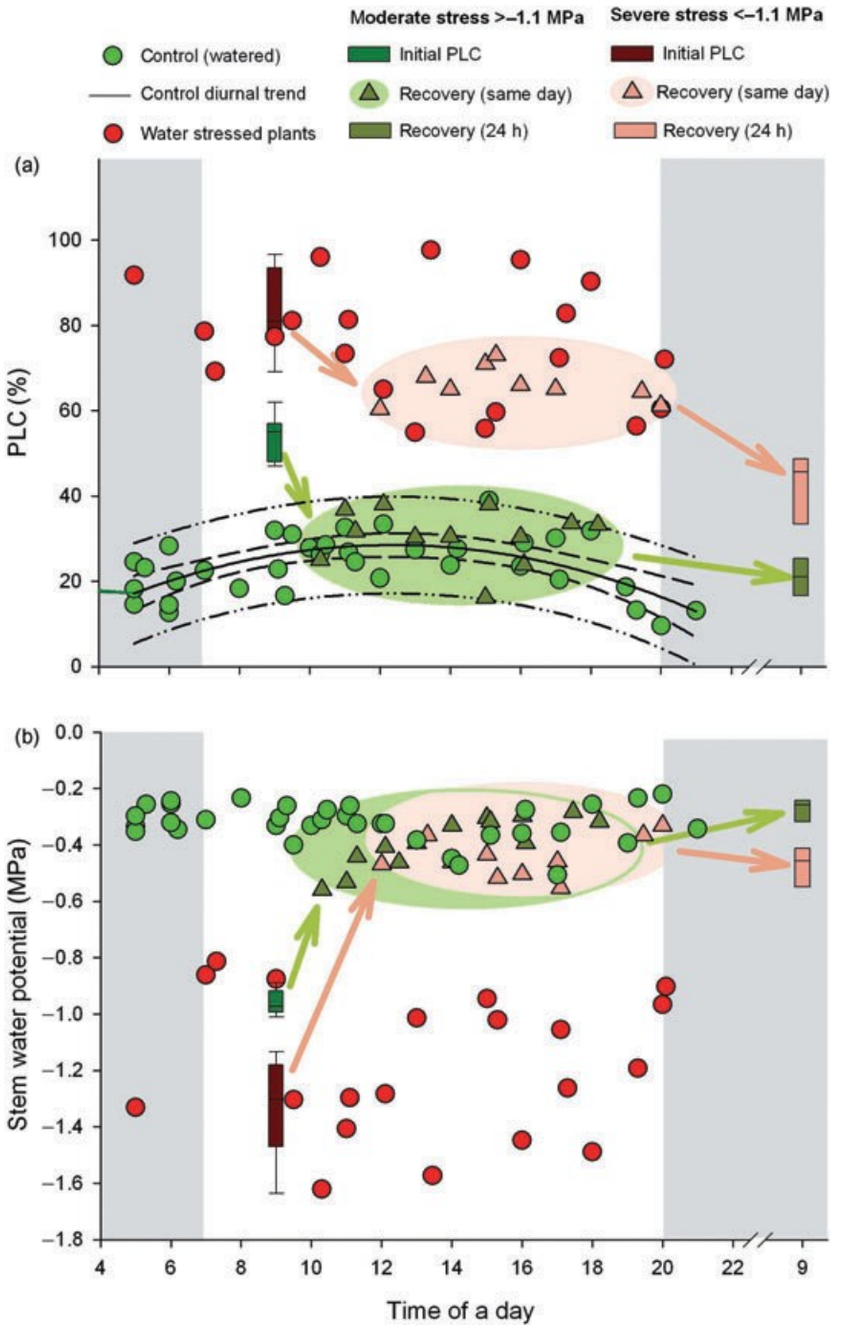


Figure 1. Susceptibility of *P. trichocarpa* stems to embolism in relation to water potential. Embolism level was determined using percent loss of conductivity (PLC) on 4 cm long stem segments and expressed versus stem water potential as determined from a non-transpiring leaf collected from that stem. Sigmoidal functions with three parameters were fitted to the vulnerability curves; each point depicts a measurement from a separate plant.

predawn PLC was in the range of 10–25% (with average PLC value = 19.5%) and this range rose to 20–40% (with average PLC value = 28.2%) by late afternoon, and then decreased during the evening to predawn values (with average PLC value = 13.6%; Fig. 2). There was a significant difference between predawn and midday PLC (*t*-test $P < 0.001$) and midday and evening PLC (*t*-test $P < 0.0001$). This pattern suggests not only that well watered plants of *P. trichocarpa* are susceptible to embolism formation during the day but also that an active process of refilling occurs while the stem water potential is negative (water is under tension) and the plants are transpiring.

The drought stress treatment resulted in a pronounced decrease in stem water potential (Y_{stem}) that was accompanied by a significant increase in PLC level. Moderately stressed plants (Y_{stem} ~ −0.9 MPa) lost hydraulic conductivity to around 60% whereas severely stressed plants (Y_{stem} ~ −1.3 MPa) had to cope with PLC losses of around 80%. Unlike control plants, there was no particular diurnal pattern of PLC in stressed plants, although we observed a very high variability in PLC among plants of similar stress levels (Fig. 2). It is interesting to notice that all plants, even those that experienced ~ −1.6 MPa, recovered from water stress within a few hours and restored low PLC over a 24 h period, thus suggesting that applied stress was not lethal.

Diurnal patterns of expression of previously characterized members of the *P. trichocarpa* PIP aquaporin family in wood tissue were measured. All eight PoptrPIP2s and five PoptrPIP1 genes were expressed in the wood of *P. trichocarpa* as reported previously by the authors (Secchi *et al.* 2009). Here, we tested whether the 13 PIP genes had specific diurnal regulation of expression level in control plants (Fig. 3). The expression pattern of the PoptrPIP2 subfamily (with the exception of *PoptrPIP2.3* and *PoptrPIP2.4*), showed a general trend characterized by an



Colour

Figure 2. Temporal change in (a) percent loss of conductivity (PLC) and (b) corresponding level of stem water potential. Diurnal trend in PLC of control plants (green circles) shows significant trend of increased PLC during midday (black line; with 95% confidence interval – dashed line and predicted values interval -dashed dotted line). Diurnal trend was fitted with quadratic function ($R^2 = 0.42$) and tested against null hypothesis of linear trend ($F = 23.66$; $P < 0.001$). Water-stressed plants that included both severe and moderate stress treatments (red circles) showed larger PLC levels but no diurnal trend, most likely because of the large variability in water stress level (b). The recovery of PLC was dependent on the degree of initial stress: with moderately stressed plants (green box plot) showing a full recovery of PLC to the level of control plants within 2 h (green triangles), whereas only partial recovery was observed for severely stressed plants (red box plot and red triangles). PLC level after 24 h of recovery is depicted by green and red box plots for moderately and severely stressed plants. A full recovery of water potential was observed within 2 h following watering in both moderately and severely stressed plants (b).

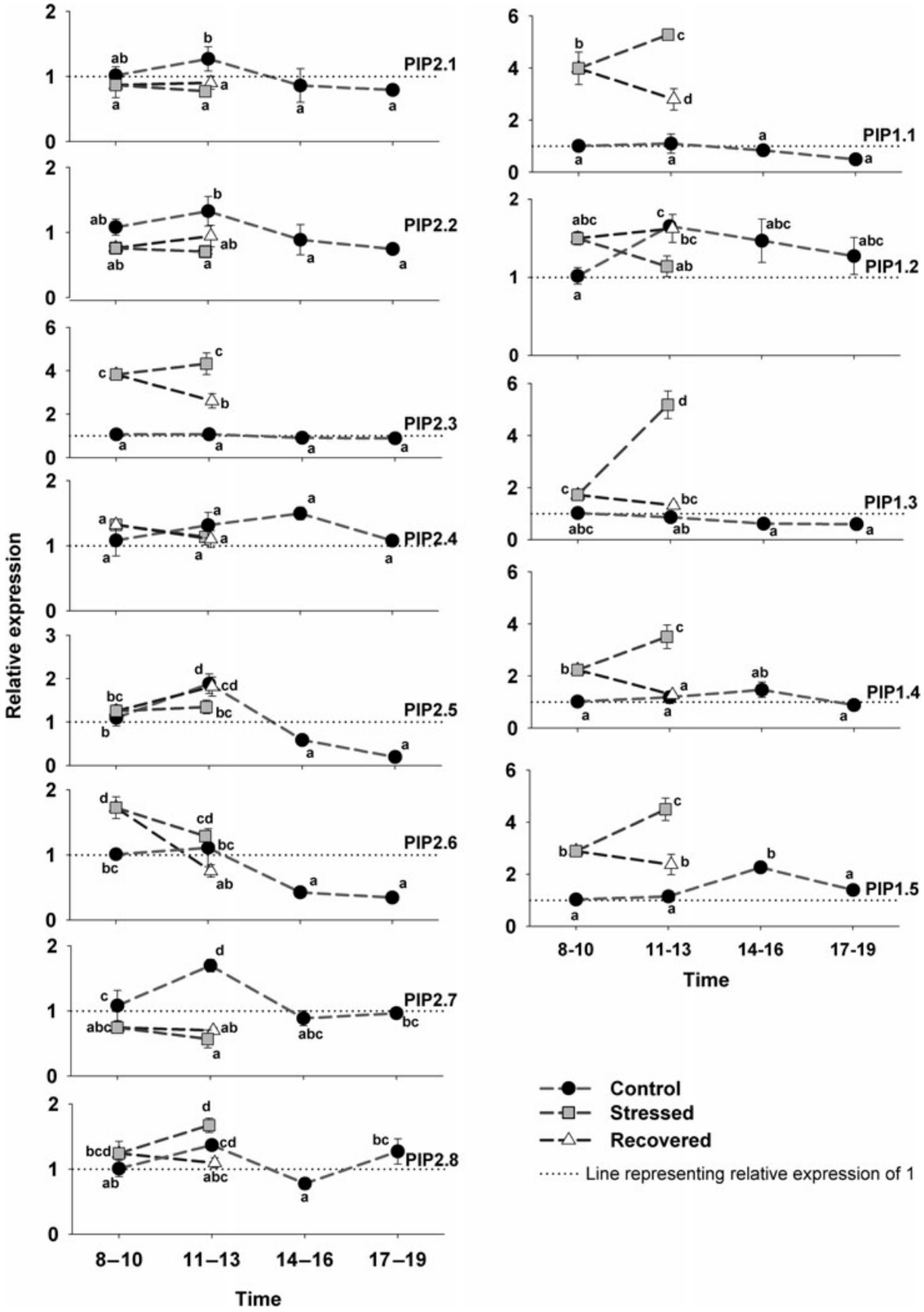


Figure 3. Relative diurnal expression of PIP2 and PIP1 aquaporins in poplar wood of well-watered (control) plants (filled green circles). Red squares denote levels of PIP expression in response to water stress and blue squares denote change of expression level in response to relief from drought conditions. Values represent the percentage of expression for each gene of the corresponding treatment with respect to that observed in control plants during the time interval 0800–1000 h. The results are the averages of three independent biological samples (error bars denote standard error). For each gene expression and analysis of variance was performed and in each case there was an overwhelming evidence that mean expression level was different in each group (with $P < 0.01$). Letters denote presence of significant difference ($P < 0.05$) from *post hoc* analysis using least significant difference method.

increase in transcript abundance in the late morning followed by a decrease in the evening, corresponding to the pattern of embolism formation (compare Figs 2a & 3). No such general trend was observed for PoptrPIP1 family where expression of most of the genes remained relatively constant during the day; *PoptrPIP1.2* and *PoptrPIP1.4* showed a small increase of expression, *PoptrPIP1.5* was the only exception showing a significant increase in transcript abundance during the day.

Moderate and severe water stress treatments had a variable and relatively small effect on the expression level of the PIP2 subfamily (Fig. 3). In general, changes were less than 2 fold with the exception of *PoptrPIP2.3*, that showed a significant increase in expression levels in response to water stress and this elevated transcript level was maintained into the early afternoon. In addition *PoptrPIP2.6* statistically differed in its gene expression in the morning but the transcript level fell back to controls by mid-day. In response to water stress, the other PIP2s showed no significant differences in the morning but in most remaining cases expression level was significantly lower during the midday, compared with the pre-stress levels (Fig. 3). By contrast, water stress had a much stronger effect on transcript accumulation of the PoptrPIP1 subfamily (Fig. 3). All genes, with the exception of *PoptrPIP1.2*, were significantly up-regulated by drought during the morning and this up-regulation was maintained or even increased during the mid-day hours. Maximum changes in expression levels were found for *PoptrPIP1.1* and *PoptrPIP1.3* that had transcript levels elevated six times higher than control plants.

Change in embolism level and gene expression upon recovery from water stress

Re-watering of stressed plants (~2 h after sunrise) resulted in a fast increase in Υ_{stem}, which returned to the value of control plants within 1 h. This relief of Υ_{stem} was not dependent on the level of initial water stress as both moderately (Υ_{stem} ~0.9 MPa) and severely stressed plants (Υ_{stem} ~1.3 MPa) showed full recovery of stem water potential (Fig. 2b). This fast recovery of Υ_{stem} was followed by recovery of PLC only in plants with moderate stress levels (Fig. 2a). In these plants, the level of PLC one hour after re-watering was similar to that of control plants for the same hour of the day, suggesting that full embolism recovery took place despite the presence of tension and transpiration. Within four hours of re-watering, severely stressed plants showed only a partial drop in PLC level and near full restoration of stem conductivity (close to the level of the control plants) did not occur until the following morning (Fig. 2a).

The recovery of Υ_{stem} and PLC corresponded to changes in the expression of PIP genes. Upon re-watering almost all PIP2 and PIP1 genes showed a strong tendency to return to expression levels similar to that of control plants (Fig. 3). This response was almost uniform across most tested genes. All genes with the initial change in expression upon water stress (*PoptrPIP2.3*, *PoptrPIP2.6* and all PoptrPIP1s, with the exception of *PoptrPIP1.2*) showed a significant drop in accumulation of the transcript in response to relief from water stress. *PoptrPIP2.3*, *PoptrPIP1.1* and *PoptrPIP1.5* followed a partial recovery while the other genes showed expression levels similar to that of control plants.

Change in embolism level and gene expression in response to artificial embolism induction

To elucidate if the changes in expression level of PIP genes were induced by water stress or by the presence of embolism, we artificially induced embolism in the stems of non-stressed plants. Embolism was induced by application of positive pressure to the stem, such that native tension together with pressure of injection was in the range of moderate stress (~ -1.0 MPa). This procedure increased native embolism from ~20% to approximately ~70% (Fig. 4). Such PLC increase was not observed in control plants that underwent identical treatment except for air injection. Within 0.5 h following air injection, a partial recovery of PLC was observed and within 2 h following the injection of air, a full recovery of PLC was observed in all tested plants (Fig. 4). The temporal dynamics of recovery from experimentally induced embolism were similar to those observed during recovery from prolonged stress except that in this case, even the most embolized plants experienced full recovery. It is important to note that stem water potential in artificially embolized stems remained constant throughout the time of the experiment and was similar to that of the control plants (~ -0.4 MPa). Such results suggest successful experimental separation of plant water status (Υ_{stem}) from embolism occurrence (PLC).

Expression of PIP genes in artificially embolized and control plants were very similar (Fig. 5). Expression levels of PIP2 genes did not significantly differ between any of the eight tested genes within 2 h of injection. Similarly, three out of five genes from the PIP1 subfamily showed little if any expression change. However, two PIP1 genes (*PoptrPIP1.1* and *PoptrPIP1.3*) showed statistically significant elevation of expression level within the first 0.5 h after induction of artificial embolism (Fig. 5). This increase of expression disappeared after 2 h coinciding with completion of refilling process. These observed changes of

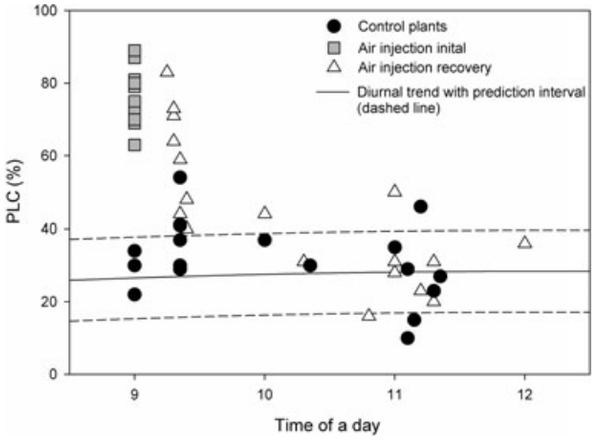


Figure 4. Temporal changes in the extent of embolisms in air-pressure injected plants and in control plants represented as percent loss of conductivity (PLC). Red circles denote the level of PLC induction and pink circles show dynamics of PLC following induction of the embolism. Control plants (green circles) were treated exactly as the injected plants including incision and chamber placement but no pressure was applied. Each point depicts a separate plant.

expression level were not caused by change in stem water potential (i.e. water stress), since air injection did not affect water status of a whole plant. Furthermore, change of expression was not caused by wounding as control plants went through the same treatments except for air pressurization. Thus, this short temporal increase of expression most likely occurred in response to embolism induction.

Comparative analysis of water channel activity and co-expression of PoptrPIP1.1 and PoptrPIP2s in *Xenopus* oocytes

Genes from the PoptrPIP2 subfamily were shown to encode functional water channels as determined by their expression in *X. laevis* oocytes (Secchi *et al.* 2009). Also, in this study all PIP2 proteins showed the ability to facilitate membrane water transport (Fig. 6). Unlike PIP2, proteins from the PIP1 subfamily showed no or little water channel activity. To further investigate the water transport function of PIP1 we focused our attention on the protein PoptrPIP1.1. This choice resulted from three cues: (1) *PoptrPIP1.1* was found to be one of the most expressed genes from PIP1 subfamily in the wood sampled (Secchi *et al.* 2009); (2) it was also initially strongly up-regulated in response to water stress and the most importantly; and (3) it was one of the two genes up-regulated in response to artificial induction of embolism. Assuming that this protein might be involved in embolism refilling by adjusting hydraulic properties of the cell membrane, we tested this protein for functionality under a variety of conditions. We measured the permeability coefficient (P_f) of *X. laevis* oocytes injected with 20 ng of cRNA encoding PoptrPIP1.1 and control oocytes injected with water, and found no significant difference between the two treatments (PoptrPIP1.1, 0.093 ± 0.017 ¥ 10^{-2} cm s^{-1} and water, 0.101 ± 0.031 ¥ 10^{-2} cm s^{-1}). This confirmed the lack of water channel activity by this protein in the functional assay (Fig. 6).

Despite the lack of water functional activity of PoptrPIP1.1, PIP1 gene expressions were elevated in response to water stress and embolism. Such results suggest that if there is a change in cell water permeability in response to stress or presence of embolism it might occur by interaction between PIP2 and PIP1, rather than solely by the change of PIP2s. Thus, we tested whether the interaction of PIP2 and PIP1 proteins can increase membrane permeability to water in *Xenopus* oocytes. For the reasons mentioned above, we used PoptrPIP1.1 to test for the presence of interaction. When PoptrPIP1.1 protein was co-expressed with proteins from the PIP2 subfamily, an increase of membrane water permeability occurred in seven out of eight tested interactions. Only co-expression of PoptrPIP1.1 with PoptrPIP2.7 did not lead to an enhancement of P_f membrane. Non-parametric analysis of all interactions strongly suggest presence of enhancement of membrane P_f when PIP1.1 gene was co-expressed with a gene from PIP2 subfamily (Wilcoxon matched pairs test with $P = 0.025063$, $n = 8$, $T = 2$ and $Z = 2.240448$ and Friedman analysis of variance and Kendall coefficient of concordance with $N = 8$, d.f. = 1 and $P < 0.03390$). Thus, we can expect adjustments in the membrane hydraulic properties of parenchyma cells during the onset of stress or embolism refilling not by changes on PIP2 protein abundance, but by changes of PIP1 expression levels and their cooperation with PIP2 proteins.

DISCUSSION

P. trichocarpa proved to be a highly vulnerable species to drought-induced cavitation, with 50% embolisms occurring around ~ -0.9 MPa and >80% PLC in the range of ~ -1.25 MPa tension. This high vulnerability is not an exception among poplar species. *P .alba* and *P. trichocarpa* ¥ *koreana* experience 50% loss of xylem conductivity when water potential is just shy of -1.5 MPa, whereas in *P. euphratica* stems reach 50% loss at around -0.70 MPa (Hukin *et al.* 2005). Such high vulnerabilities reflect an inability to resist drought and may suggest that even a small degree of water stress will lead to massive cavitation and death. Although it is generally true that *P. trichocarpa* thrives in mesic conditions, it also true that in such mesic environments, there are short-term drought events and often stem water potential can drop below -1.0 MPa. Thus, we have to assume that embolism formation is a common occurrence in *P. trichocarpa*. If this is so, one can expect that the long-term outcome of such conditions would be the accumulation of embolized vessels over the growing season – which does not appear to occur. Thus, one has to assume that daily embolism formation is counteracted by embolism refilling (Canny 1997; Zwieniecki & Holbrook 1998). This study provides support for the notion of a continuous diurnal physiological cycle of embolism formation and refilling in *P. trichocarpa*. This was especially

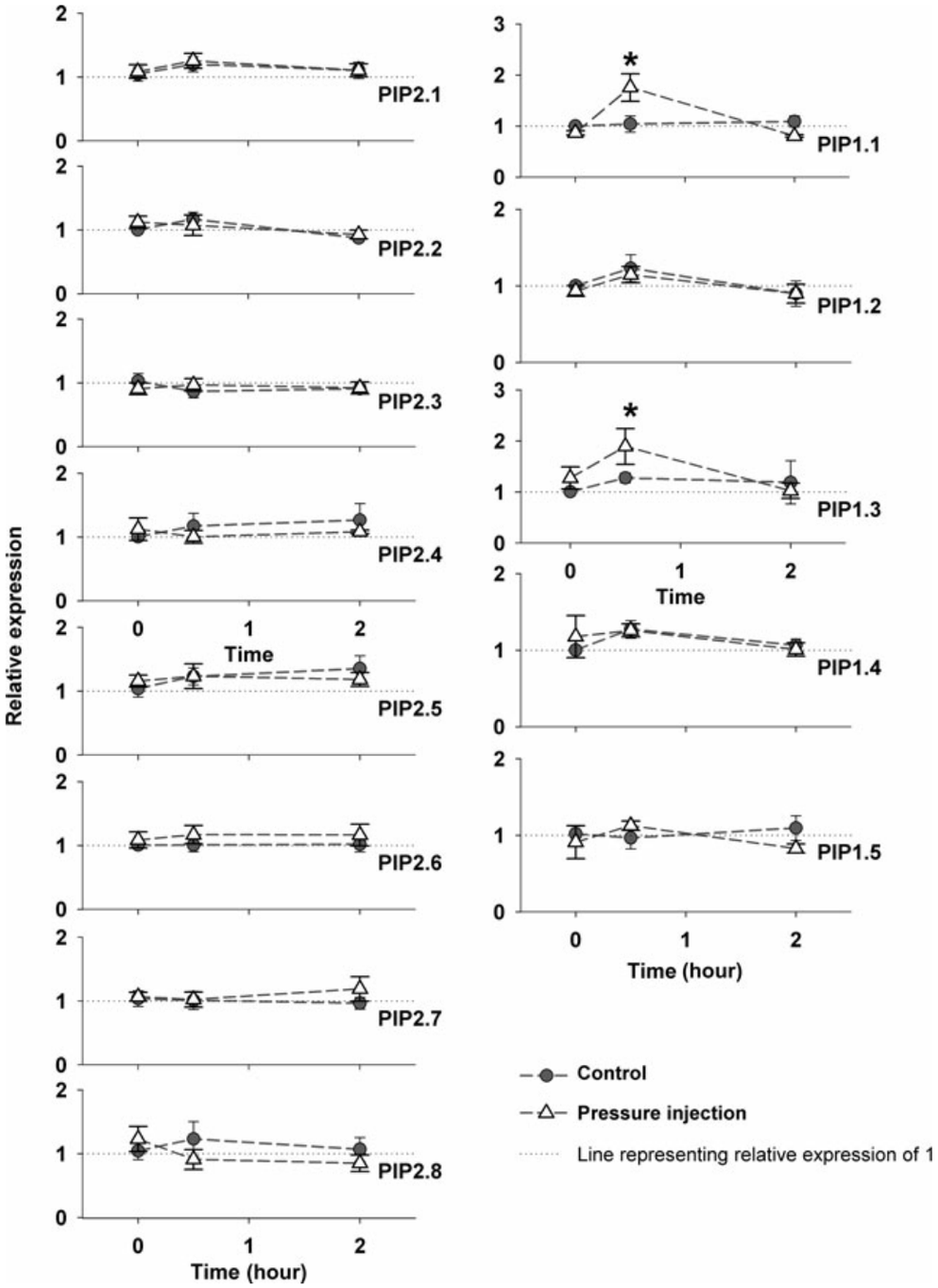


Figure 5. Relative expression level of PIP2 and PIP1 genes in response to artificially induced embolism in the stems of non-stressed plants at time of air injection and from pooled wood samples after ~0.5 and ~2 h following the air injection (red circles). The transcript levels of each PIP of induced embolized plants were plotted as the relative expression of the control plants (green circles) at the time of air injection. The results are the averages of two independent biological samples extracted from pooled wood material collected from a minimum of three plants per treatments (error bars denote standard error). For each gene expression and analysis of variance was performed. An evidence exists that mean expression level were different ($P < 0.01$) only for PIP1.1 and PIP1.3. Stars denote presence of significant difference in expression level ($P < 0.05$) from post-hoc analysis using least significant difference method.

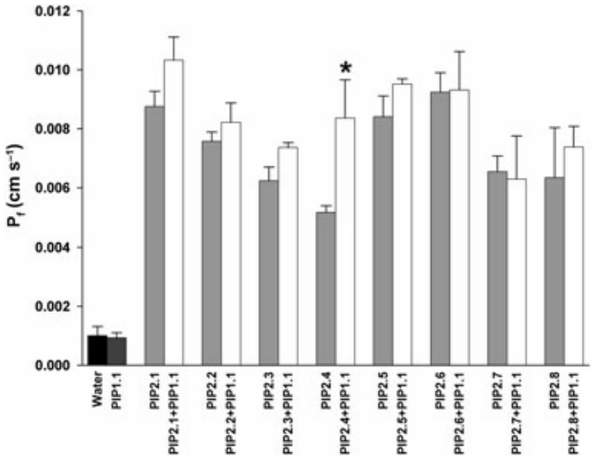


Figure 6. Functional activity of PIP2s and PIP1.1 in *Xenopus laevis* oocytes. Osmotic water permeability coefficient values (P_f) of oocytes that express PIP2s and/or PIP1.1 were measured. Oocytes were injected with 20 ng of cRNA encoding aquaporin or with the same volume of water (control) 3 d before the assay. The results are the means of 10–12 measurements; vertical bars denote standard errors. Star denotes significant difference within the pair of measurements ($P < 0.05$).

the case for well-watered plants, where diurnal changes in PLC varied by 20% with the highest losses recorded around midday. This diurnal trend suggests that *P. trichocarpa* is capable of embolism refilling during active transpiration and low water stress.

The picture gets somewhat more complicated when we look at the plants experiencing moderate to severe water stress. Percent loss of conductivity was higher and more variable under stress. This variability could be the reason for observed lack of diurnal pattern in PLC which was seen in the control plants. In such adverse conditions repair might be absent or scarce (i.e. repair cannot keep up with the embolism formation). Such high levels of PLC are not uncommon, as drought often leads to increased PLC in nature (Bucci *et al.* 2003; Melcher *et al.* 2003; Lovisolo *et al.* 2008), but surprisingly these high PLC levels rarely lead to death of the plant. In fact, plants often recover to pre-stress status following a single rain event (Zwieniecki & Holbrook 1998). It was previously shown that the ability of a stem to refill is not common to all poplar species (e.g. *P. euphratica* did not recover from drought-induced embolism, at least over a day period) (Hukin *et al.* 2005). However, we found that water-stressed *P. trichocarpa* plants were able to recover from water stress and conductivity loss after re-watering. The tension relief following rehydration was almost instantaneous in both moderately and severely stressed plants; while recovery from stress-induced embolism showed two distinct patterns that depended on initial level of stress. Moderately stressed plants, with native PLC around 55%, returned to the level of PLC of well-watered plants within the first hour following plant rehydration. Severely stressed plants, with native PLC around 80%, recovered only a fraction of PLC following several hours after rehydration, and near full recovery of embolism was observed only the following morning. These findings indicate that plants are capable of dealing with moderate embolism over very short temporal scales despite active transpiration and presence of low tension. However, recovery from sever embolism levels requires a prolonged decrease in transpiration rates such as during the night.

Successful refilling requires that energy and water sources are simultaneously available (Holbrook & Zwieniecki 1999). Previous studies suggested that phloem might be a source of the water necessary for refilling (Sakr *et al.* 2003). Since there is a physical separation of phloem from vessels, xylem parenchyma cells can be seen as bridges that allow water to flow from phloem to embolized conduit. This pathway involves multiple crossing of cellular membranes and thus it would be mediated by the activity of water channels. In this study, we monitored the expression of plasma membrane aquaporins in relation to diurnal trends of embolism-refilling cycle. This cycle in well-watered plants did not correspond to any dramatic diurnal trend of aquaporin expression levels of either water channel subfamilies (PoptrPIP1 and PoptrPIP2). Only a small increase in midday expression was observed in several genes of PIP2 subfamily. Lack of dramatic diurnal changes in PIP gene expression in the woody part of the stem differs from those reported for leaves and roots where strong diurnal trends were observed (Moshelion *et al.* 2002; Lopez *et al.* 2003; Beaudette *et al.* 2007; Hachez *et al.* 2008). This discrepancy might be associated with the relatively constant stem water status relative to water potentials and level of embolism experienced by leaves. In addition, aquaporins may play a different role in the woody part of plants. In distal parts, aquaporins are responsible for connecting the environment to the plant and for regulating large changes in uptake and evaporative demand. In wood, water flows via dead xylem vessels and the contribution of parenchyma cells might be vital only in the case of an emergency when a radial connection between xylem and phloem is necessitated (i.e. high stress or recovery from massive embolism events) thus explaining the lack of change in gene expression in the wood of control plants.

Studies of PIP gene expressions to water stress showed highly variable responses including up-, down- or no regulation (Alexandersson *et al.* 2005; Vandeleur *et al.* 2009). Different range in responses might be species or tissue specific, associated with stress level or its length, or could depend on the specific physiological role of each PIP's gene isoform. Currently it is very difficult to provide a general pattern of aquaporin gene expression in response to stress. The present results add another interesting pattern of expression specific to stem of a woody plant during onset of drought stress, stress recovery and embolism formation.

In this study, water stress treatments resulted only in a very small effect on the expression level of functional aquaporins PIP2 subfamily, with an exception of the *PotrPIP2.3* and *PotrPIP2.6* genes. This finding initially suggested a very limited change in adaptation of stem parenchyma cells to stress via adjustment of membrane hydraulic properties.

However, a very strong and positive effect on expression level was observed in all of the PIP1 subfamily genes (with the only exception of *PoptrPIP1.2*) despite their lack of water channel activity when expressed in the membrane of the oocytes, as was proved here for *PoptrPIP1.1* or tested for maize PIP1 proteins (Chaumont *et al.* 2000). The discrepancy between no change in the expression of functional aquaporins and the large adjustments in non-functional ones may be resolved if there is a positive interplay between PIP1.1 and PIP2 proteins. Co-expression of PIP1.1 protein with each member of poplar PIP2 subfamily led to an increase in membrane permeability over expression of a single PIP2 protein. Such positive interaction in oocytes membranes was also reported among the members of PIP proteins belonging to other plant species (Fetter *et al.* 2004; Temmei *et al.* 2005; Mahdieh *et al.* 2008). However it is important to emphasize that the observed lack of water transport activity of PIP1.1 gene could result from its inability to be functionally expressed in *Xenopus* membranes. However, no matter the reason for the lack of transport activity of PIP1 proteins in oocytes observed in this study, when expressed together with PIP2 proteins they increased membrane water permeability. As PIP1 proteins are always being co-expressed with PIP2 proteins in poplar stem, we can speculate that stress adaptation in the form of membrane permeability is not achieved in *P. trichocarpa* via direct change in the expression level of PIP2 proteins but rather via significant changes in PIP1 proteins and their interplay with PIP2 group. Upon re-watering, most of PIP2 and PIP1 genes showed a strong tendency to return to the expression levels of control plants within 2–3 h after water application. As in the case of stress response, the PIP1 subfamily was strongly affected by the re-watering treatment – again showing the important role of the PIP1 proteins in the rapid adjustment of membrane hydraulic properties in response to plant water status. Therefore, the PIP1 subfamily proteins seem to play an important role in drought response in woody plant stems and potentially be involved in embolism/refilling cycle.

Can plants sense embolism as a separate stimulus to water stress? This question is highly important to the understanding of the biology of the refilling processes. The experimental induction of embolism in non-stressed plants was undertaken here to explicitly separate the effect of embolism on gene expression from the general water stress effect. In all cases where embolism was induced artificially, the stem water potential remained at control plant levels, thus effectively isolating the embolism stimulus from that of general water stress. A small level of refilling was already detected 0.5 h after the stem pressurization and full recovery was present within 1.0 h of the treatment. The corresponding expression pattern of the PIP2 subfamily genes showed no change between control and pressurized plants. However, we found significant up-regulation of two PIP1 genes in response to induction of embolism (*PoptrPIP1.1* and *PoptrPIP1.3*). This increased expression was only observed during the recovery from embolism within the first hour after the induction. Two hours later, when the embolism level returned to its initial value, the expression level of both PIP1 genes returned to the level observed in control plants. Again, as in the case of the stress response, embolism presence triggered only changes in the PIP1 subfamily but not PIP2, suggesting the important role of these proteins in the maintenance of xylem water transport in the stems of poplar. What is even more surprising was that the observed effect suggested the existence of a trigger that can recognize presence of the embolism separately from changes in water potential. Of course, we do not argue that embolism is a direct trigger of the expression of PIP genes. However, our data suggest that embolism is recognized by the living cells, and this most likely can happen via sudden change in the water status of the parenchyma cells. The nature of this trigger is not yet known. We believe that changes induced by the air injection and natural embolism may be very similar since a sudden switch from the functional to embolized status generates a transient pressure change of the same magnitude: −0.3–0.7 MPa (~1.0 MPa) in the case of the injection and −1.0 to ~0 MPa (~1.0 MPa) in the case of natural embolism. Such a large change in the apoplastic water potential in the vicinity of the parenchyma cells might trigger a response to start the refilling process.

Although this research is only an initial step in our quest to understand refilling under tension from a molecular perspective in woody plant, it already provides some important clues for a better understanding of linkage between changes of aquaporin expression and plant response to water stress and embolism refilling:

1. Expression levels of the PIP2 subfamily do not change in response to water stress or to embolism presence in xylem of *P. trichocarpa*, although a small level of diurnal expression pattern with up-regulation around midday was present.
2. In contrast to PIP2 genes, genes of the PIP1 subfamily showed high up-regulation of expression levels in response to water stress, while no diurnal pattern was observed.
3. Two genes *PoptrPIP1.1* and *PoptrPIP1.3* were up-regulated in response to induction of embolism without the presence of water stress and were down-regulated soon after refilling occurred.
4. Up-regulation of specific genes in response to the presence of embolism within 0.5 h after embolism induction suggests that there is a physiological/molecular mechanism allowing for fast detection of embolism in xylem that does not include changes in general plant water status.
5. Patterns of PIP gene expression in xylem cells suggest that changes in membrane water permeability are mediated by PIP1 proteins and not by the PIP2 subfamily during drought and refilling process in *P. trichocarpa*.

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