Down-regulation of Plasma Intrinsic Protein1 Aquaporin in poplar trees is detrimental to recovery from embolism

PIP1s contribute to embolism recovery

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Down-regulation of PIP1 aquaporin in poplar trees is detrimental to recovery from embolism.

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Abstract

During their life cycles trees encounter multiple events of water stress that often result in embolism formation and temporal decreases in xylem transport capacity. The restoration of xylem transport capacity requires changes in cell metabolic activity and gene expression. Specifically, in poplar, the formation of xylem embolisms leads to a clear up-regulation of PIP1 aquaporin genes. To determine their role in poplar response to water stress, transgenic *Populus tremula x Populus alba* plants characterized by the strong down regulation of multiple isoforms belonging to the PIP1 subfamily were used. Transgenic lines demonstrated that they are more vulnerable to embolism with 50% PLC occurring 0.3 MPa earlier than in wild type plants and that they also have a reduced capacity to restore xylem conductance during recovery. Transgenic plants also show symptoms of a reduced capacity to control PLC via stomatal conductance in response to drought as they have a much narrower vulnerability safety margin. Finally, a delay in stomatal conductance recovery during the period of stress relief was observed. The presented results suggest that PIP1 genes are involved in the maintenance of xylem transport system capacity, promote recovery from stress, and contribute to a plant's control of stomatal conductance under water stress.

Introduction

Long-distance water transport in vascular plants occurs in a conduit network of nonliving cells connecting roots to leaves (Sperry, 2003). Often under drought conditions, the water column within the lumen of xylem vessels or tracheids can be subjected to tensions that result in cavitation and the subsequent formation of embolisms (Holbrook and Zwieniecki, 2008). This hydraulic failure within the xylem network can cause tissue damage, loss of plant productivity and, ultimately, plant death (Tyree and Sperry, 1989; Sperry et al., 1998; Zwieniecki and Holbrook, 2009). Plants have evolved several strategies to prevent and/or mitigate the effects of hydraulic failure due to embolism and to restore xylem transport capacity once embolism occurs (Stiller and Sperry, 2002; Nardini et al., 2011; Secchi and Zwieniecki, 2012). These strategies include passive, often long-term, responses like the growth of new vessels/tracheids or dieback followed by the growth of new shoots (shrubs) or active, often fast, responses that result in the restoration of hydraulic failure by the formation of root pressure (Cochard et al., 1994; Ewers et al., 1997; Yang et al., 2012) or stem activity (embolism removal), (Salleo et al., 2004; Nardini et al., 2011; Brodersen and McElrone, 2013).

While embolism formation is a purely physical process related to the degree of tension in the water column and to a wood's physicochemical properties (Brennen, 1995; Tyree and Zimmermann, 2002), embolism removal requires that empty vessels fill with water against existing energy gradients as the bulk of water in the xylem remains under tension due to transpiration. Thus, recovery from embolism cannot happen spontaneously and necessitates some physiological activities that promote water flow into embolized vessels (Holbrook and Zwieniecki, 1999; Tyree et al., 1999; Salleo et al., 2004; Zwieniecki and Holbrook, 2009; Secchi et al., 2011). Visual evidence from cryo-SEM studies, MRI observations and CT-scans showed that water (xylem sap) can return to empty vessels, suggesting that plants do have the ability to restore functionality in the xylem (Holbrook et al., 2001; Clearwater and Goldstein, 2005; Scheenen et al., 2007). Brodersen and colleagues showed that water droplets preferentially form on the vessel walls adjacent to parenchyma cells and that these droplets grow until the lumen completely refills (Brodersen et al., 2010). In addition, scientific support for the existence of embolism/refilling cycles in intact stems of *Acer rubrum* are provided using magnetic resonance imaging (Zwieniecki

et al., 2013). Droplet formation on the walls of empty vessels that are in contact with parenchyma cells support predictions that these living cells supply both water and energy to drive the restoration of xylem hydraulic function.

Processes related to water transport across the cellular membrane involve plasma intrinsic protein (PIP- aquaporins) moderators and thus the role of PIPs must be considered when contemplating how plants recover from embolism formation. Plant aquaporins show a great diversity and are classified into five major homologous groups that reflect specific subcellular localizations (Prado and Maurel, 2013). Among different aquaporin gene families [NIP, TIP, XIP, SIP, PIP; (Danielson and Johanson, 2008)], the PIPs represent the largest number of members and can be further divided into two subfamilies, PIP1 and PIP2. There is a large body of evidence that aquaporins from the PIP2 subfamily contribute to water transport. The generation of data has been multidisciplinary and involved the use of chemical blockers, the down and up regulation of genes in plants and the expression of these proteins in oocytes (Hukin et al., 2002; Postaire et al., 2010; Shatil-Cohen et al., 2011). Expression levels of several PIP and TIP members change following the dynamic of increasing water stress and recovery in many woody plants including walnut, poplar and grapevine (Sakr et al., 2003; Secchi et al., 2011; Perrone et al., 2012; Laur and Hacke, 2013; Pou et al., 2013). Further, an increase in the expression of PIP2.1 and PIP2.2 genes was observed in vessel-associated parenchyma cells in walnuts at the same time that recovery from embolism was taking place (Sakr et al., 2003). The role of genes from the PIP1 subfamily in tree responses to water stress is less well understood. PIP1s were shown to have little to no water channel activity when expressed in oocytes on their own. However, co-expression of PIP1.1 proteins with an isoform from the PIP2 subfamily led to higher membrane permeability than that observed with the expression of a single PIP2 protein (Fetter et al., 2004; Secchi and Zwieniecki, 2010). With respect to their role in mediating water stress, it was shown that the expression level of several PIP1 genes in poplar changed significantly during the onset of stress, during recovery, during the formation of embolisms following water stress, and under no stress conditions but with induced embolism, while the expression of PIP2 genes remained mostly unresponsive (Secchi and Zwieniecki, 2010; Secchi et al., 2011; Secchi and Zwieniecki, 2011).

Despite significant effort invested in elucidating the contribution of aquaporins to the regulation of xylem hydraulic capacity throughout the progression of drought and recovery from water stress, evidence of their active role *in vivo* is only partially confirmed. Genetic approaches provide a reliable and effective strategy for determining the physiological function of aquaporin genes in plant water relations. However, most studies thus far have been conducted on herbaceous plants (Kaldenhoff et al., 1998; Postaire et al., 2010). For example, *Arabidopsis thaliana* plants expressing PIP antisense genes exhibit an impaired ability to recover from water stress (Martre et al., 2002) and knock-out mutants exhibit reduced leaf hydraulic conductivity (Da Ines et al., 2010). NtAQP1 down-regulated tobacco plants show reduced root hydraulic conductivity and lower water stress resistance (Siefritz et al., 2002). RNA technology, although not often used for woody plants, has been adapted for grapevine (Perrone et al., 2012) and for *Eucalyptus* trees (Tsuchihira et al., 2010); in both cases, analysis focused on over-expressing specific isoforms of aquaporin genes. The PIP2;4 root-specific aquaporin enhanced water transport in transformed *Vitis* plants under well-watered conditions but not under water stress (Perrone et al., 2012), while *Eucalyptus* hybrid clones over-expressing RsPIP1;1 and RsPIP2;1 did not display any increase in drought tolerance (Tsuchihira et al., 2010). Up till now, no research on the recovery from embolism formation in woody plants with impaired aquaporin expression has been conducted.

In the presented study, we used poplar transgenic plants characterized by a strong down-regulation of PIP1 genes to test the role of this aquaporin subfamily in the plant response to water stress and subsequent recovery from stress. While transformed poplars did not show morphologically different phenotypes when compared to wild-type plants, they were found to be more sensitive to imposed water stress resulting in increased vulnerability to embolism formation and the loss of stomatal conductance. We also noted a reduced capacity of transformed plants to restore xylem water transport.

Results

Physiological changes in response to water stress

Populus tremula x alba transformed trees were previously generated using a reverse genetic approach (RNAi) aimed at suppressing more than one gene belonging to the poplar PIP1 subfamily (Secchi and Zwieniecki, 2013). Silencing the entire sub-family was preferred to silencing particular isoforms in order to avoid the potential for compensation of expression within that same gene group. To estimate levels of PIP1 subfamily down-regulation in the stems of five selected transgenic lines, RT-PCR analyses were performed. The expression of PIP1 genes was strongly reduced in all lines examined when compared to the wild-type (*wt*), decreasing by 91-94% (Figure 1, ANOVA $p<0.001$). Since there were no significant differences in PIP1 expression level among lines (Figure 1), we pooled all lines into a single transformed group in order to increase the sample size in subsequent physiological experiments. Once pooled, analysis yielded a 93% reduction in expression for PIP1 subfamily gene compared to wild-type (*wt*), (Figure 2, *t*-test; $p<0.001$). Additionally, PIP1.1 and PIP1.3 genes [two highest expressed genes in stems from PIP1 subfamily (Secchi et al., 2009) and the most responsive genes to drought and embolism formation (Secchi and Zwieniecki, 2010)] showed reduced expression levels in the combined transgenic stems confirming the successful down regulation of multiple isoforms belonging to the same subfamily. The expression of the other genes belonging to PIP1 subfamily was also monitored. All PIP1 genes were strongly down-regulated and resulted to be significant different from their expression in stems of *wt* plants (Figure 2 and Supplemental Online Material Figure S1A). To test the possible compensatory response of the PIP2 gene subfamily members in response to PIP1 down-regulation, the transcript levels of seven out of eight genes belonging to PIP2 subfamily were measured (PIP2.8 was analyzed but it expression was not detected in the stem tissue). In general, their gene expressions were not significantly different from *wt* plants suggesting a lack of PIP2 compensatory response (Figure 2 and Online Material Figure S1B).

Both *wt* and PIP1 down-regulated poplars showed similar levels of native embolism in well-watered plants averaging around 30.7% and 37.3%, respectively (Figure 3A). An increase in water stress resulted in additional losses of stem hydraulic conductivity plateauing above 80%, below xylem pressures (P_{xylem}) of -2.3 MPa in both groups. The relationship between the loss of hydraulic conductivity and xylem pressure was fitted using four-parameter logistic curves (dose response curves – see methods). The 50% loss of effective stem conductivity (not including

native embolism) described by the $EC50_{PLC}$ parameter of the curve (half maximal *'effective concentration'* - in our case *'effective xylem pressure'*) occurred at −1.76 MPa (SE = 0.0642, t = 27.3493, p<0.0001) for *wt* plants and at −1.43 MPa (SE = 0.0816, t = 17.54, p<0.0001) for transgenic plants. The $EC50_{PLC}$ parameter was significantly different between the two groups [Z-test; t = 3.120, df = 47, p < 0.0025; (Paternoster et al., 1998)], indicating that the stems of transgenic lines were more vulnerable to embolism than the stems of *wt*. For the purpose of clarity we will later refer to plants as moderately stressed when xylem pressure is above $EC50_{PLC}$ (i.e. PLC<50%, but below xylem pressure of well watered plants) and severely stressed if xylem pressure is below $EC50_{PLC}$ (i.e. PLC>50%).

Stomatal conductance (g_s) was similar for non-stressed (well watered) *wt* and PIP1 down-regulated plants (~ 600 mmol m^{-2} s^{-1}), (Figure 3B). A decrease in g_s was observed with an increase in water stress, and both groups showed full stomatal closure at xylem pressures below −2.0 MPa. Changes in stomatal conductance in response to xylem pressure were fitted with the 'dose-response' curve. An effective drop in g_s by 50% from its observed maximum in well-watered plants ($EC50_{gs}$) was at -1.102 MPa for *wt* and at -1.316 MPa for transgenic lines. There was a statistical difference between $EC50_{gs}$ parameters of the two groups (Z-test; t = 2.066, df = 43, p < 0.025). Importantly, *wt* plants closed their stomata by 50% at approximately ~0.6 MPa before $EC50_{PLC,}$ providing a relatively wide PLC safety margin, while transgenic plants closed stomata at only ~0.1 MPa ahead of $EC50_{PLC}$, providing a very narrow safety margin. However, both groups completely closed their stomata before the maximum loss of stem conductance occurred.

The total osmotic potential (estimated from combined concentration of sugars and ions) of sap collected from functional vessels was mostly accounted for by the presence of ions (Figure 4). Ion impact on osmotic potential of xylem sap was ten times higher than sugar content in xylem sap collected from well watered and moderately stressed plants, and four times higher for severely stressed plants (see Supplemental Figure 2). Osmotic potential under no stress conditions was not different between *wt* and transgenic lines. Osmotic potential increased with the increase of water stress in both groups reaching 0.125 MPa and 0.089 MPa, respectively, for

wt and transgenic trees. There was no significant difference in sap osmotic potential in moderately stressed plants and only a small increase in osmoticum content in *wt* plant over transgenic under severe stress (Figure 4).

Physiological changes upon recovery from induced water stress

Moderately and severely stressed plants were re-watered to their field capacity and allowed 1.5 hours of recovery time (Figure 5). Re-watering moderately stressed plants resulted in a fast increase of xylem pressure (P_x) in both *wt* and transgenic lines. P_x returned to the values of non-stressed control plants within the allotted time (Figure 5). This relief of P_x was correlated with the restoration of xylem transport capacity for *wt* plants that showed almost full embolism recovery (95.45% initial embolism incidence). However, only partial PLC recovery (43%) was observed in PIP1 down regulated lines despite recovery of P_x. The recovery from embolism was significantly different between *wt* and transgenic plants (t-test; $t = 2.1150$, $df = 17$, $p < 0.05$, see Supplemental Table S1). Recovery from severe stress was not different between *wt* and transgenic trees. Both groups showed a significant recovery of xylem pressure and both groups showed only a partial drop in the level of PLC, which was especially low in plants recovering from a drop in P_x below -2.0 MPa (Supplemental Online Material Table S1).

Direct observation of plant recovery from stress using time-lapse imaging showed that turgor recovery in leaves could be characterized by two phases; (1) a slow phase – that lasted for 37-40 minutes – and was characterized by a slow steady decrease in the angle between the petiole and the stem and (2) a fast phase – that lasted more than 40 minutes – characterized by a fast change in the angle that resulted in total recovery of initial leaf position (Figure 6). The rate of recovery in the slow phase was similar in both groups of plants. The rate of recovery during the fast phase was only similar in plants recovering from low stress levels above $EC50_{PLC}$ (P_x was less negative than $EC50_{PLC}$). However, recovery from stress around $EC50_{PLC}$ or lower (P_x was equal to or more negative then $EC50_{PLC}$) was significantly slower in transgenic plants resulting in a delay of several minutes in restoration of turgor and pre-stress leaf positions (Figure 6).

The recovery of stomatal conductance did not follow the patterns of fast recovery observed in P_x (minutes to a couple of hours; Figure 7), leaf turgor (Figure 6) and PLC (Figure 5). Full g_s recovery did not occur until after 4 days of full irrigation. The general pattern of g_s recovery was also different in plants recovering from moderate stress and severe stress between *wt* and transgenic plants. Moderate stress only forced full stomata closure in *wt* plants while transgenic stomata remained partially open (Figure 7; and compare Figure 3B). Wild-type moderately stressed plants showed signs of some g_s recovery immediately after re-watering (first day - Figure 7 B and C). The recovery was not observed in transgenic plants despite their tendency to have higher initial (under stress) g_s. The recovery of g_s continued in *wt* plants during the second day, reaching the g_s of control, non-stressed plants during the third day. Full recovery occurred four days after the return of irrigation. Transgenic plants did not show significant signs of g_s recovery in the second day but recovery was similar to *wt* plants in third and fourth day. Severe stress (below $EC50_{PLC}$) forced full stomatal closure in both *wt* and transgenic plants. Recovery from severe water stress did not start during the first day for either plants group, but later showed a similar pattern to that observed in recovery from moderate stress. Again, the start of the partial recovery of g_s in transgenic lines was delayed one day, when compared to *wt* (Figure 7 D-E). Differences of g_s values between mornings and afternoons were related to variation in greenhouse temperature (Figure 7A).

The osmotic potentials (sugars and ions) of sap collected from functional vessels during recovery from stress are accounted for by ion concentration with little contribution from sugar content (Supplemental Figure 3). Wild-type plants recovering from moderate and severe stress showed osmotic potential values similar to those measured in well-watered plants (Figure 8, grey bars), with the exception of higher osmotic values found under severe stress. Liquid collected from moderately and severely stressed transgenic plants had lower osmoticum concentrations than control plants (Figure 8, white bars), suggesting that these plants may be impaired in their utilization of ions in their response to stress.

Discussion

The significant role that PIP1 aquaporins play in stem response to both presence of water stress (Secchi and Zwieniecki, 2010) and artificially induced embolism in the xylem (Secchi and Zwieniecki, 2011) has been previously suggested. However, these studies provided association based only on changes in PIP gene expression in response to particular treatments. The presented approach of comparative response analysis between *wt* and transgenic plants, with down-regulated expression of multiple isoforms of the PIP1 subfamily, directly points at the role that PIP1 genes play in two basic physiological functions: stem hydraulics and stomatal conductance during the onset of water stress. We also show that the dynamics of recovery from water stress are significantly affected by lower levels of PIP1 expression.

Stems of transgenic poplars showed a substantial reduction in PIP1 gene expression in all selected lines (Figure 1). The pooled transformed group did not show a significant compensatory regulation effect of PIP2 expression in response to PIP1 down-regulation, while different isoforms belonging to the PIP1 subfamily were strongly down-regulated (Figure 2). Despite the substantial suppression of gene expression, down-regulating the PIP1 transcript did not affect the basic physiological functions of non-stressed trees. Both *wt* and transgenic plants had similar stomatal conductance and stem hydraulic properties including similar levels of native xylem embolism (Figure 3A). This lack of phenotypic or functional impairment due to the down-regulation of PIP1 genes coincides with a previous report showing that both plant groups did not significantly differ in physiological functions related to photosynthesis (Secchi and Zwieniecki, 2013). This finding is also in agreement with other studies; data reported for transgenic banana plants (constitutively overexpressing a PIP1;2 gene) showed that they were phenotypically and physiologically indistinguishable from the untransformed lines under normal growth conditions (Sreedharan et al., 2013). A similar behavior was also reported by Siefritz et al., (2002) evidencing that, despite a strong reduction in NtAQP1 expression and changes in root hydraulic conductivity, tobacco plants grown under optimal conditions in the greenhouse did not show morphological changes.

The physiological similarity of *wt* and transgenic plants did not persist under water stress conditions. Transgenic lines were more susceptible to xylem embolism displaying a 50% loss of

PLC at less negative xylem pressure (-1.4 MPa vs -1.7 MPa for transgenic and *wt* plants, respectively), indicating that the presence of PIP1 genes might be beneficial to plant vulnerability resistance (Figure 3A). This increased vulnerability to embolism in transgenic plants was associated with their reduced capacity to control stomatal conductance during stress development, suggesting a different content of abscissic acid (ABA) in the tissues and consequently a delay of stomata closure in response to drought stress. In transgenic plants, 50% of stomatal shutdown occurred at -1.3MPa and in *wt* plants, at -1.1 MPa, with approximately 40% of maximum g_s at $EC50_{PLC}$ for transgenic plants and less than 10% of maximum g_s at $EC50_{PLC}$ for *wt* plants (Figure 3B). Since stomatal closure is one of the mechanisms that plants can adopt in order to limit water loss and control stress level, the behavior assumed by transgenic plants suggests that they were less likely to control transpiration rates to protect xylem from embolism formation. In other words, the down regulation of PIP1 gene expression resulted in a significant reduction of the xylem vulnerability safety margin (Sperry and Ikeda, 1997; Choat et al., 2012; Johnson et al., 2012).

As embolism formation is mostly a physical process it might be hard to imagine how PIP1 genes are able to influence a plant's vulnerability curve without also affecting morphology or xylem anatomy. However, previous studies have shown a positive correlation between PIP1 expression and stress conditions, as well as the up-regulation of PIP1 expression in response to embolism formation even in the absence of water stress (Secchi and Zwieniecki, 2010). Thus, in combination with results presented here we can propose that the processes of embolism formation and refilling are not separated in time but happen simultaneously with respective rates that are functionally linked to plant stress level. Embolism formation rates are expected to increase with increases in stress while embolism refilling rate are expected to decrease with stress. To rephrase, the current level of PLC at any given moment is a result of embolisms formed minus refilling. As the down-regulation of PIP1 genes negatively affects refilling rate, the result of the competition between the two processes shifts transgenic plants towards an apparently higher vulnerability to embolism formation while this is in fact a result of reduced capacity to refill. Thus, PIP1s do not directly influence cavitation thresholds but do reduce refilling rates.

Re-watering moderately stressed plants resulted in the fast recovery of xylem pressure in both groups of plants and was followed by full or partial recovery of xylem hydraulic conductivity. Full recovery of hydraulic capacity to the initial PLC was observed in *wt* plants recovering from moderate stress, while the xylem PLC recovery of transgenic plants was significantly impaired. PLC recovery was in the range of ~44% within one and a half hours' time, indicating that only *wt* plants were capable of dealing with moderate embolism incidence over short temporal scales despite active transpiration and the presence of tension. Recovery from severe water stress was similar between *wt* and transgenic plants; both groups recovered a large fraction of their initial xylem pressure but only a small fraction of their initial PLC in the allotted time (Figure 5). This impaired recovery of PLC observed in transgenic plants might be partially related to the lower xylem sap osmotic potential that was found in both plants under severe water stress conditions and in plants recovering from stress (Figure 4 and 8). While the concentration of sap collected from functional vessels is very low and cannot be used to explain the driving force required for recovery (Secchi and Zwieniecki, 2012), it can reflect the ability of plant xylem parenchyma cells to move solutes to vessels, thus suggesting that *wt* plants were more capable of loading vessels with solutes than transgenic plants. The observed delay in recovery of stem hydraulic parameters (several hours) in transgenic poplar was relatively small compared with reports of slow recovery observed in the transgenic *Arabidopsis* plants with down-regulated PIP genes. A delay of several days was observed for the recovery of hydraulic conductance and transpiration rates of transgenic plants returning from stress when compared to *wt* plants (Martre et al., 2002).

The dynamics of recovery from water stress in terms of P_x and PLC did not coincide with the recovery of stomatal conductance. Although *wt* plants recover significantly faster during the first two days following re-watering than transgenic plants, a full recovery did not occur until four days of full irrigation (Figure 7). This pattern of PLC recovery but delayed g_s recovery from drought is a common phenomenon and has been observed in *Eucalyptus pauciflora*; stem hydraulic capacity was restored within six hours but stomatal conductance had not fully recovered even ten days after a return to favorable water status (Martorell et al., 2013). Similar results were reported for grapevines; xylem embolism in petioles, roots and shoots recovered

during the 24 hours following rehydration while stomatal conductance required an additional 48 hours (Lovisolo et al., 2008). Evidence of a delay in g_s recovery from drought suggests that the regulation of stomatal conductance depends on factors beyond the supply of water via the xylem and stem water pressure, possibly the functionality/integrity of the photosynthetic system and/or abscisic acid physiology (Lovisolo et al., 2008; Brodribb and McAdam, 2013). Thus the delayed g_s recovery in transgenic plants might imply that the regulation of aquaporin expression may involve an ABA-dependent signaling pathways (Wan et al., 2004).

The function of PIP1 genes in promoting tolerance to water stress has been studied through reverse genetic approaches that over or under express aquaporin genes (Martre et al., 2002; Cui et al., 2008; Kaldenhoff et al., 2008; Zhang et al., 2008; Postaire et al., 2010; Sreedharan et al., 2013). Conclusions have been made with antisense tobacco plants; under well-watered conditions the NtAQP1 aquaporin did not seem to be important for water uptake or management. However, in a water limited environment NtAQP1 antisense plants were not able to maintain turgor and appeared to be less drought tolerant when compared with wild-type tobacco plants (Siefritz et al., 2002). When subjected to drought, tobacco plants with overexpressed BnPIP1 were more tolerant to water stress, while the antisense lines with reduced mRNA levels of BnPIP1 showed reduced water uptake and were more sensitive to water deficiency (Yu et al., 2005). Results presented here suggest that the major effect of the PIP1 gene subfamily on the stress physiology of woody plants is directly linked to plant management of apparent vulnerability to embolism (Secchi and Zwieniecki, 2010; Secchi and Zwieniecki, 2012). Specifically, we propose here that the observed increased vulnerability to embolism formation and the significant delay in recovery of hydraulic capacity in transgenic plants indicates that a loss of PIP1 gene expression reduces the rate of refilling and effectively shifts the balance between the rates of embolism formation and refilling such that embolism became a dominate process at lower tensions. Apparent increased of vulnerability without obvious phenotypic differences between *wt* and transgenic plants underlines the role of physiology in the maintenance of xylem hydraulic capacity and suggests that continuous competition between the processes of embolism formation and removal might be mediated by PIP1 activity (Zwieniecki and Holbrook, 2009; Zwieniecki et al., 2013).

Material and Methods

Plant materials and experimental design

Wild-type (*wt*) and transgenic hybrid white poplars (*Populus tremula x Populus alba*, INRA-France clone 717-1B4) were used for the study. Down-regulated PIP1 transgenic plants were previously generated and described in Secchi and Zwieniecki (2013). Poplars were grown in a greenhouse with the following ambient conditions: temperature maintained in the range of 25°C to 32°C and natural daylight supplemented with light from metal halogen lamps to maintain a minimum of 500–600 µmol photons m^{-2} s^{-1} during 12/12-h light/dark cycle. Plants were approximately 1.5 m tall at the onset of the experiments.

A total of 124 poplars were used (56 *wt* and 64 transgenic plants) in four different experiments:

1. *Poplar response to increasing water stress*. Fifty-three poplars (25 *wt* and 28 transgenic plants) were used in this study, of these 12 plants were kept as controls and they were watered every day to field capacity. The remaining 41 plants were gradually subjected to drought by stopping irrigation. Plants were used to construct PLC curve and relate stomatal conductance to xylem pressure. Duration of drought treatment depended on the levels of desired water stress, and it was between one and five days. Physiological measurements (PLC, xylem pressure and g_s) were performed between 9 am to 12 pm.
2. *Dynamics of plants recovery from embolism*. Thirty five plants (14 *wt* and 21 transgenic) were water stressed and then re-watered in the morning (~9 am). Plants were allowed 1.5 hours of recovery time followed by measurements of PLC and xylem pressure.
3. *Dynamics of stomatal conductance recovery*. Twenty stressed plants (11 *wt* and 9 transgenic) were re-watered in the morning (~9 am) to field capacity and the dynamic of stomatal conductance recovery was monitored. Stomatal measurements started just prior the re-watering (~9 am) and continued until 3 pm during four consecutive days. Plants were irrigated several times during a day.
4. *Dynamics of plants recovery from wilting (movies).* Sixteen plants (8 *wt* and 8 transgenic) were used. One *wt* and one transgenic plant were grown in the same 5.7 x 8.3 cm pot. Plants were allowed to wilt in the pots over the period of several days. When the desired wilting point was achieved plants were re-watered and subsequent recovery observed. The

temporal dynamics of leaf movements during increasing water stress and recovery were recorded using time-lapse video. Pictures were taken during water stress development every 5 minutes, and every 30 seconds during the recovery period. Analysis of leaf motion was performed every hour during stress development and every 6 minutes during the recovery period.

Expression of aquaporin genes in transgenic poplars

Total RNA was isolated from *wt* and transgenic stems according to the protocol of Chang, (Chang et al., 1993). First strand cDNA was synthesized from total RNA treated with DNase I (Fermentas) using oligo$(dT)_{12–18}$ as primers (Fermentas) and SuperScript II Reverse Transcriptase (Invitrogen). The sequences of primers used for Real time PCR analysis are listed in the Table S2. Primers were tested on cDNA of hybrid poplar through PCR with RED Taq DNA Polymerase (Sigma) according to the manufacturer's instructions. The transcript abundance of each gene was quantified with SYBR Green JumpStart Taq Ready Mix (Sigma) on an Eco Real-Time PCR System (Illumina, San Diego, USA). Thermo-cycler conditions for all real-time analyses were: 95ºC for 5 min, followed by 40 cycles of 95ºC for 15 s, 60ºC for 30 s, and 72ºC for 30s.

Data were analyzed using Eco software (Illumina), and the expression values were normalized to the geometric mean of two housekeeping genes (ubiquitin and actin). These genes were found to have, for the same poplar species, the highest amplification efficiency and most stable expression across different tissue (Carraro et al., 2012). Real-time PCR was carried out using three biological replicates per transformed line. Two technical replicates were performed for each of the three biological replicates.

Measurements of xylem pressure and stem hydraulic conductivity

Stem water pressure was measured on non-transpiring leaves. Leaves were covered with aluminum foil and placed in a humidified plastic bag for 15 minutes prior to excision. After excision, leaves were allowed to equilibrate for an additional 20 minutes before the xylem pressure was measured using a Scholander-type pressure chamber (Soil Moisture Equipment Corp., Santa Barbara, CA, USA).

Stem hydraulic conductivity was measured using a standard approach previously described (Secchi and Zwieniecki, 2010). Briefly, 1.5m long shoot was cut under water. Within few minutes this initial cut was followed with cutting a set of set of 3 stem segments. Segments were excised under water, approximately 20-30 cm from the initial cut (distance longer than 2x length of vessels in studied poplar). Each segment was approximately ~4 cm long. The initial hydraulic conductance (k_i) of each stem segment was measured gravimetrically by determining the 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. During measurements stems were submerged in a water bath with a water level ~10 cm below the level of water on the balance. After a steady flow rate was reached (within a few minutes), the tube connecting the stem to the balance was closed, and a bypass tube was used to push water across the segment under ~ 0.2 MPa of pressure for approximately 20 s to remove embolism. The chosen segment length used for determining PLC was short enough to have the majority of vessels open in poplar stems (vessel length is usually ~5cm), thus making removal of embolism very easy and complete within few seconds. Stem conductance was then re-measured to find maximum conductance (k_{max}). The percent loss of conductance (PLC) was calculated as $PLC = 100 * (k_{max}-k_i)/k_{max}$. The same procedure was used in experiment 1 and 2.

Measurements of stomatal conductance

Stomatal conductance was measured using SC-1 Leaf Porometer (Decagon Devices, Pullman, WA) on fully expanded leaves. Gs values on control and water stressed plants (*wt* and transgenic) were measured between 9 am and 12 am with increasing water stress and from 9 am to 3 pm during recovery from stress (experiment 1 and 3). Along the process several leaves were collected for estimation of stem water pressure.

Curve fitting

Relationships between percent loss of conductance (PLC) and stomatal conductance (g_s) in response to stem water pressure were fitted with a four-parameter logistic curve (dose response); where: $PLC=initial_{PLC}+(maximum_{PLC}- initial_{PLC})/(1+(P_{xylem}/EC50_{PLC})^{Slope_{EC50PLC}})$, and $g_s=minimum_{gs}+(intial_{gs}-minimum_{gs})/(1+(P_{xylem}/EC50_{gs})^{Slope_{EC50gs}})$. This function was

preferred over other sigmoidal shapes as it allows treating xylem pressure as a treatment (dose) and allows for the fit of initial values to true preexisting conditions. $EC50_{PLC(gs)}$ is the parameter describing a 50% change in the curve between the initial value of PLC or g_S and the corresponding final value at very low xylem pressures (PLC-maximum and g_s-minimum). $Slope_{PLC(gs)}$ describes the rate of change in PLC or g_s at the inclination point of the curve. In order to compare *wt* and transgenic plants PLC and g_s response to xylem water pressure, we compared $EC50_{PLC(gs)}$ parameters of fitted curves using the corrected statistical Z-test for the equality of regression coefficients (Paternoster et al., 1998).

Carbohydrate and ion contents in xylem sap of functional vessels

The xylem sap of functional vessels was collected from the same plants that were used to determine PLC and xylem pressure (experiment 1 and 2) using the procedure previously described (Secchi and Zwieniecki, 2012). Briefly, leaves were removed and the stem was attached through a plastic tube to a syringe needle. The needle was threaded through a rubber cork to a vacuum chamber with the needle tip placed in the 1.5 ml plastic tube. After the generation of a vacuum, short pieces of stem were consecutively cut from the top allowing liquid from open vessels to be sucked out of the stem and collected in the tube. Collected liquid was then analyzed for sugar and ion concentrations following the procedures described in detail by (Secchi and Zwieniecki, 2012). Briefly, carbohydrate content was quantified using the colorimetric anthrone-sulfuric acid assay (Leyva et al., 2008). 50 µl of xylem sap were added to 150 µl of fresh anthrone reagent, samples were mixed, kept 10 min at 4 °C and then incubated 20 min at 100 °C. After heating, they were cooled for 20 min at room temperature and absorbance at 620 nm was read with a microplate multiscan reader (Multiscan Thermo Scientific). Total carbohydrate content was calculated as mg/ml of glucose, and from the deduced molal concentration of each xylem sap solution the relative osmotic potential was calculated based on the law for perfect gases: $\Pi = miRT$. *W*here: m = molality of the solution (moles of solutes/1000g H_20), i = a constant that accounts for ionization of the solute, for glucose $i = 1$, R = the gas constant (0.00831 liter MPa mol^{-1} K^{-1}) and T = temperature, 293.16 K.

Ion concentration was measured as electrical conductivity using a 5 µl capillary fitted with gold electrodes at the both ends and connected to a multi-meter (True RMS digital multimeter 289; Fluka Europe). Liquid samples were sucked into the capillary using a pipettor. A series of potassium chloride solutions with different concentrations was used to establish a calibration curve. Electrical conductivity of xylem sap was translated to the equivalent concentration of potassium ions.

Movies on drought

Digital images taken from movies (experiment 4) were analyzed using ImageJ software (http://rsbweb.nih.gov/ij/). For each plant at least 2 leaves were selected and the angle between stem axes and arm, described as a line linking leaf blade base and petiole base, was measured on a series of consecutive pictures during the increasing of water stress and upon recovery from stress. Dynamics of the stress development required measurements at one hour intervals while recovery from stress was much faster and required measurements at 6 minute intervals.

Supplemental data

The following materials are available in the online version of this article.

- Supplemental Figure S1. Relative gene expression in the stems of each transgenic line analyzed.
- Supplemental Figure S2. Sugar (A) and ion (B) osmotic potentials collected from the xylem sap of plants (*wt*, light red; transgenic, light green) subjected to increased water stress (xylem pressure).
- Supplemental Figure S3. Sugar (A) and ion (B) osmotic potential collected from xylem sap of well-watered, stressed and recovered plants.
- Supplemental Table S1. Mean values (± SD) of xylem pressure and % of PLC recovered for moderately and severely stressed *wt* and transgenic plants
- Supplemental Table S2. Sequences of primers used for quantitative real time PCR

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Figure legends

Figure 1

Relative gene expression of PIP1 subfamily in the stems of *wt* and five transgenic lines (1-5). Each histogram is the average of three independent biological samples with two technical replicates; the error bars represent SE. The one way Anova test suggests significant differences between plant groups ($P < 0.001$). Letters denote homogeneous groups based on the Fisher LSD test; no differences were observed among the transformed plants (1-5).

Figure 2

Relative expression levels of PIP1 subfamily gene (transgenic construct), PIP1s (1-5) and PIP2s (1-7) genes for pooled transgenic P. *alba x tremula* stems tested against expression level in *wt* stems. Data are mean values and the error bars represent SE. Letters denote homogeneous groups based on the Fisher LSD corrected for the multiple comparisons. Anova test revealed the presence of significant differences for all PIP1 genes tested ($p < 0.001$), while no differences were founded among all PIP2 genes tested in transgenic when compared to *wt* plants.

Figure 3

A) Percent loss of hydraulic conductance (PLC) in stems, and B) stomatal conductance (g_s) of *wt* and transgenic lines in relation to xylem pressure.

Data were fitted with the four-parameter logistic curves ('dose response curve', full lines for *wt*; dotted/dash black lines for transgenic) in the form of: PLC=minPLC+(maxPLC-minPLC)/(1+(Ψ/$EC50_{PLC}$)^slope)), where minPLC was the minimum PLC in well-watered plants, maxPLC was 100%, $EC50_{PLC}$ representing a 50% loss of initial functionality [(minPLC+(maxPLC-minPLC)] (half maximal 'effective concentration' - in our case 'effective xylem pressure'), and slope the rate of PLC increase at $EC50_{PLC}$. Same function was used to fit the g_s response to stem water pressure. Red circles/dash line and green triangles/dash line represent $EC50_{PLC}$ for $_{wt}$ and transgenic plants, respectively; while red and green stars/full lines represent a 50% loss of initial g_s ($EC50g_s$) for *wt* and transgenic plants, respectively. Parameters that describe curves for the two population of plants are statistically different (*wt* $EC50_{PLC}$ = -1.756 and

719 transgenic $EC50_{PLC}$ = -1.432; t-test, $p < 0.0025$; $_{wt}$ $EC50g_s$ = -1.102 and transgenic $EC50g_s$ = -
720 1.316, t-test, $p < 0.025$).
721
722 **Figure 4**
723 Changes in osmotic potential (sugar + ion) of xylem sap collected from functional vessels (*wt*,
724 lightly colored circles; transgenic, lightly colored triangles) under different levels of xylem
725 pressure (balancing pressure). Dark circles (*wt*) and dark triangles (transgenic) represent average
726 values for three groups of plants well watered, moderately ($EC50_{PLC}$<50%) and severely
727 ($EC50_{PLC}$>50%) stressed plants. The one way Anova test suggests significant differences between
728 treatments and lines (P <0.001). Letters denote homogeneous groups based on the Fisher LSD test.
729
730 **Figure 5**
731 PLC and xylem pressure recovery from moderate ($EC50_{PLC}$ <50%) and severe ($EC50_{PLC}$ >50%)
732 water stress levels for *wt* (A), and transgenic (B) plants occurring within 1.5 hours following re-
733 watering. Black- and white-filled symbols represent the predicted values of PLC for severely and
734 moderately stressed plants, respectively, and were calculated based on measured xylem pressure
735 and the parameters of vulnerability curves. Red (*wt*) and green (transgenic) circles represent plants
736 recovering within 1.5 hours from severe water stress, light red (*wt*) and light green (transgenic)
737 circles show recovery from moderate stress. Dashed lines indicate $EC50_{PLC}$ = 50%.
738
739 **Figure 6**
740 The rate of recovery from wilting in plants exposed to different levels of water stress expressed
741 in degrees per minute change of the angle between stem and line connecting petiole attachment
742 to the stem and leaf blade base (A). Data were collected from eight videos with transgenic and
743 *wt* plant in each video. Statistical analysis revealed a significant difference in the slope during the
744 second phase (fast phase) between *wt* and transgenic plants (*wt* =1.029; transgenic = -0.725; t-
745 test; t = -2.317 df = 10, p<0.05). Inserts: (B) Typical changes in the angle measured during
746 gradually increasing water stress (hours) and during recovery after re-watering (minutes). The
747 dotted line indicates the time of re-watering. (C) The temporal dynamic of recovery is composed
748 of two phases—a slow phase and a fast phase. (D) Visualization of the angle between stem and

749 line connecting petiole attachment to the stem and leaf blade base. The angle was measured
750 every hour under increasing water stress and every 6 minutes during recovery.
751
752 **Figure 7**
753 Temporal dynamics of the recovery of stomatal conductance (g_s) and xylem pressure in plants
754 recovering from moderate (B-C) and severe (D-E) water stress for *wt* (black bars) and transgenic
755 (grey bars) lines. Measurements were conducted over four consecutive days in a greenhouse
756 conditions. (A) provides mean values of greenhouse temperatures.
757 Stressed plants were re-watered the first day of the experiment a few minutes after 9 am, the time
758 when xylem pressure and g_s values were measured [*wt*, netted pattern (green) bars; transgenic,
759 cross pattern (yellow) bars]. Dashed lines show g_s and xylem pressure for both *wt* and transgenic
760 well-watered controls plants [there was no difference between *wt* and PIP1 down-regulated
761 controls for both g_s and xylem pressure; dashed lines are mean values ± SD (shaded areas)]. One
762 way Anova test suggests significant differences between morning and afternoon greenhouse
763 temperatures ($p< 0.001$), g_s ($p< 0.001$) and xylem pressure ($p< 0.001$) in plants recovering from
764 moderate and severe stresses. Letters denote homogeneous groups based on the Fisher LSD
765 method (lower-case letter, *wt*; upper-case letter, transgenic lines). Bars are mean values and
766 error bars represent SD.
767
768 **Figure 8**
769 Total osmotic potential collected from stem xylem sap of plants recovering from moderate
770 ($EC50_{PLC} < 50\%$) and severe ($EC50_{PLC} > 50\%$) water stress. A one way Anova test suggests
771 significant differences between treatments in both *wt* and transgenic plants ($p< 0.001$). Letters
772 denote homogeneous groups based on the Fisher LSD method (lower-case letter, *wt*; upper-case
773 letter, transgenic lines). Bars are mean values and error bars represent SD.
774
775

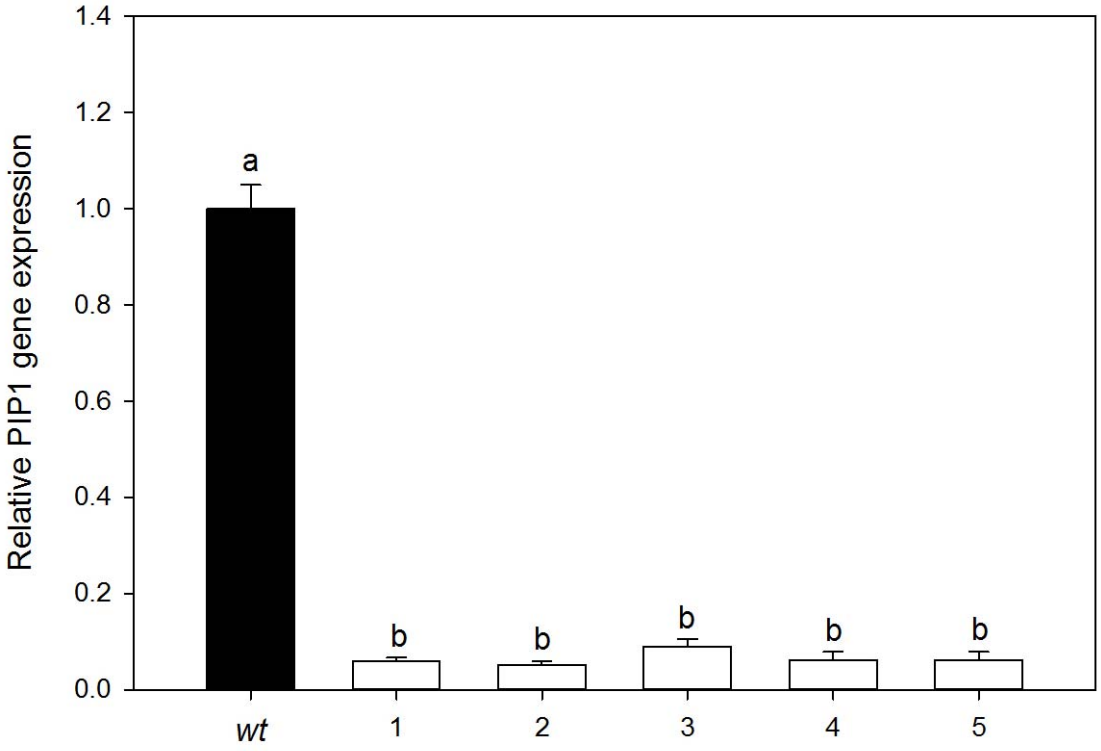

Figure 1

Relative gene expression of PIP1 subfamily in the stems of *wt* and five transgenic lines (1-5). Each histogram is the average of three independent biological samples with two technical replicates; the error bars represent SE. The one way Anova test suggests significant differences between plant groups ($P < 0.001$). Letters denote homogeneous groups based on the Fisher LSD test; no differences were observed among the transformed plants (1-5).

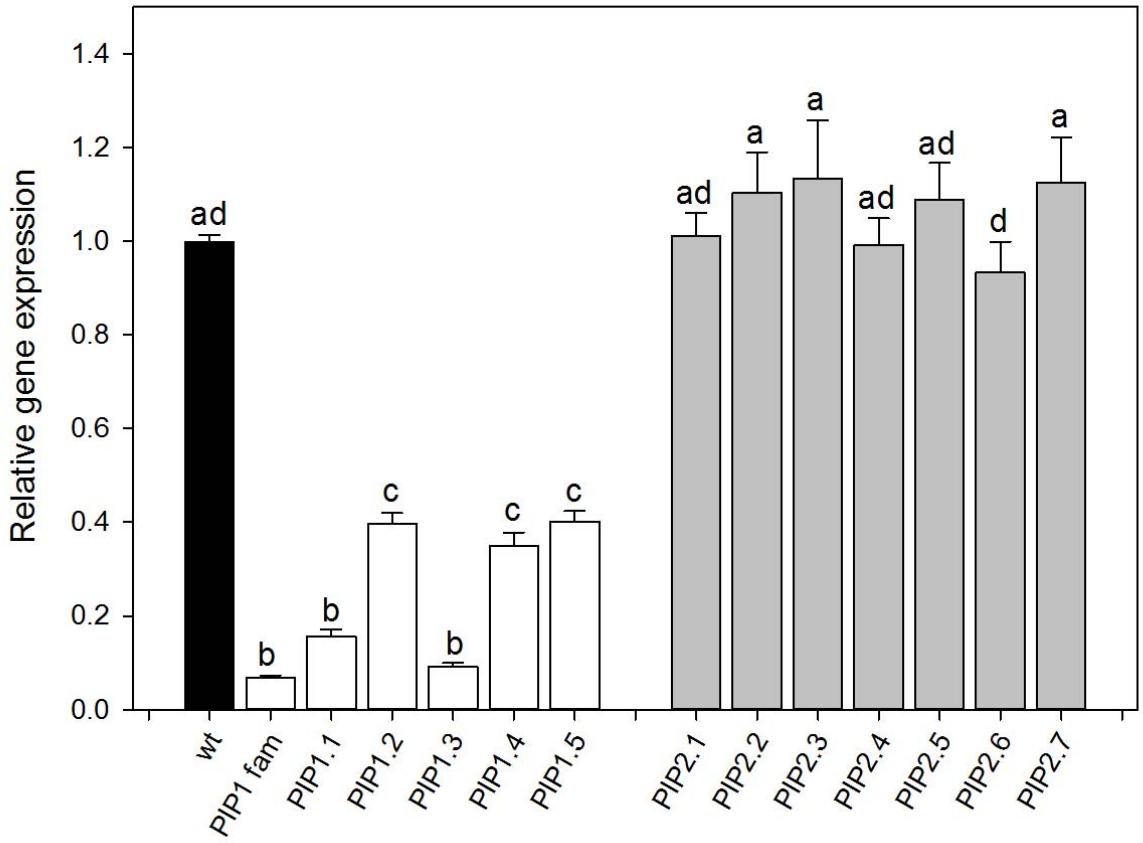

Figure 2

Relative expression levels of PIP1 subfamily gene (transgenic construct), PIP1s (1-5) and PIP2s (1-7) genes for pooled transgenic *P. alba x tremula* stems tested against expression level in *wt* stems. Data are mean values and the error bars represent SE. Letters denote homogeneous groups based on the Fisher LSD corrected for the multiple comparisons. Anova analysis revealed the presence of significant differences for all PIP1 genes tested ($p < 0.001$), while no differences were founded among all PIP2 genes tested in transgenic when compared to *wt* plants.

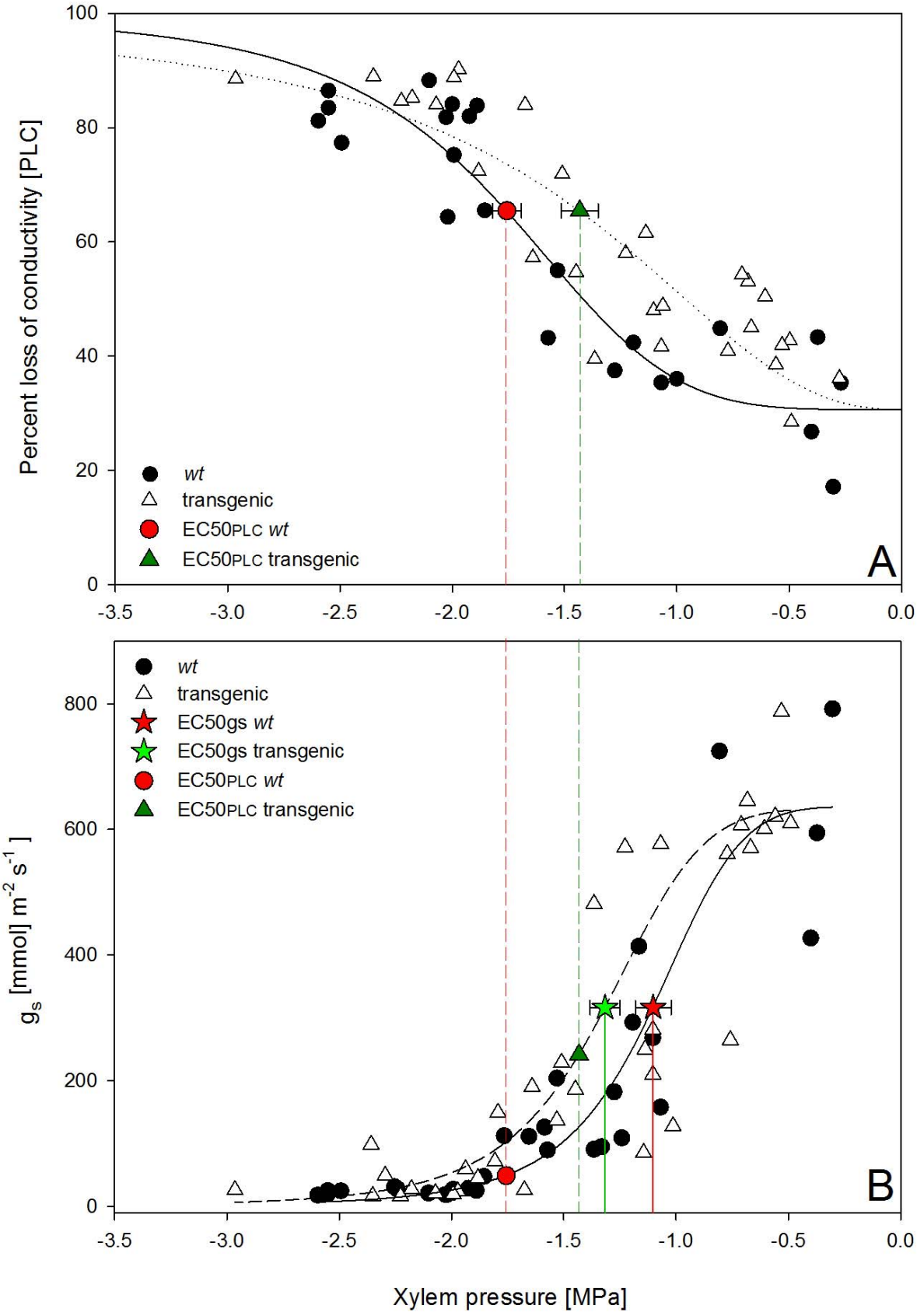
Percent loss of conductivity [PLC]
100
80
60
40
20
0
wt
transgenic
EC50PLC wt
EC50PLC transgenic
A
-3.5
-3.0
-2.5
-2.0
-1.5
-1.0
-0.5
0.0
gs [mmol] m-2 s-1]
800
600
400
200
0
wt
transgenic
EC50gs wt
EC50gs transgenic
EC50PLC wt
EC50PLC transgenic
B
Xylem pressure [MPa]

Figure 3

A) Percent loss of hydraulic conductance (PLC) in stems, and B) stomatal conductance (g_s) of *wt* and transgenic lines in relation to xylem pressure.

Data were fitted with the four-parameter logistic curves ('dose response curve', full lines for *wt*; dotted/dash black lines for transgenic) in the form of: PLC=minPLC+(maxPLC-minPLC)/(1+(Ψ/$EC50_{PLC}$)^slope)), where minPLC was the minimum PLC in well-watered plants, maxPLC was 100%, $EC50_{PLC}$ representing a 50% loss of initial functionality [(minPLC+(maxPLC-minPLC)] (half maximal *'effective concentration'* - in our case '*effective xylem pressure'*), and slope the rate of PLC increase at $EC50_{PLC}$. Same function was used to fit the g_s response to stem water pressure. Red circles/dash line and green triangles/dash line represent $EC50_{PLC}$ for *wt* and transgenic plants, respectively; while red and green stars/full lines represent a 50% loss of initial g_s ($EC50_{gs}$) for *wt* and transgenic plants, respectively. Parameters that describe curves for the two population of plants are statistically different (*wt* $EC50_{PLC}$ = -1.756 and transgenic $EC50_{PLC}$ = -1.432; t-test, $p < 0.0025$; *wt* $EC50_{gs}$ = -1.102 and transgenic $EC50_{gs}$ = -1.316, *t*-test, $p < 0.025$).

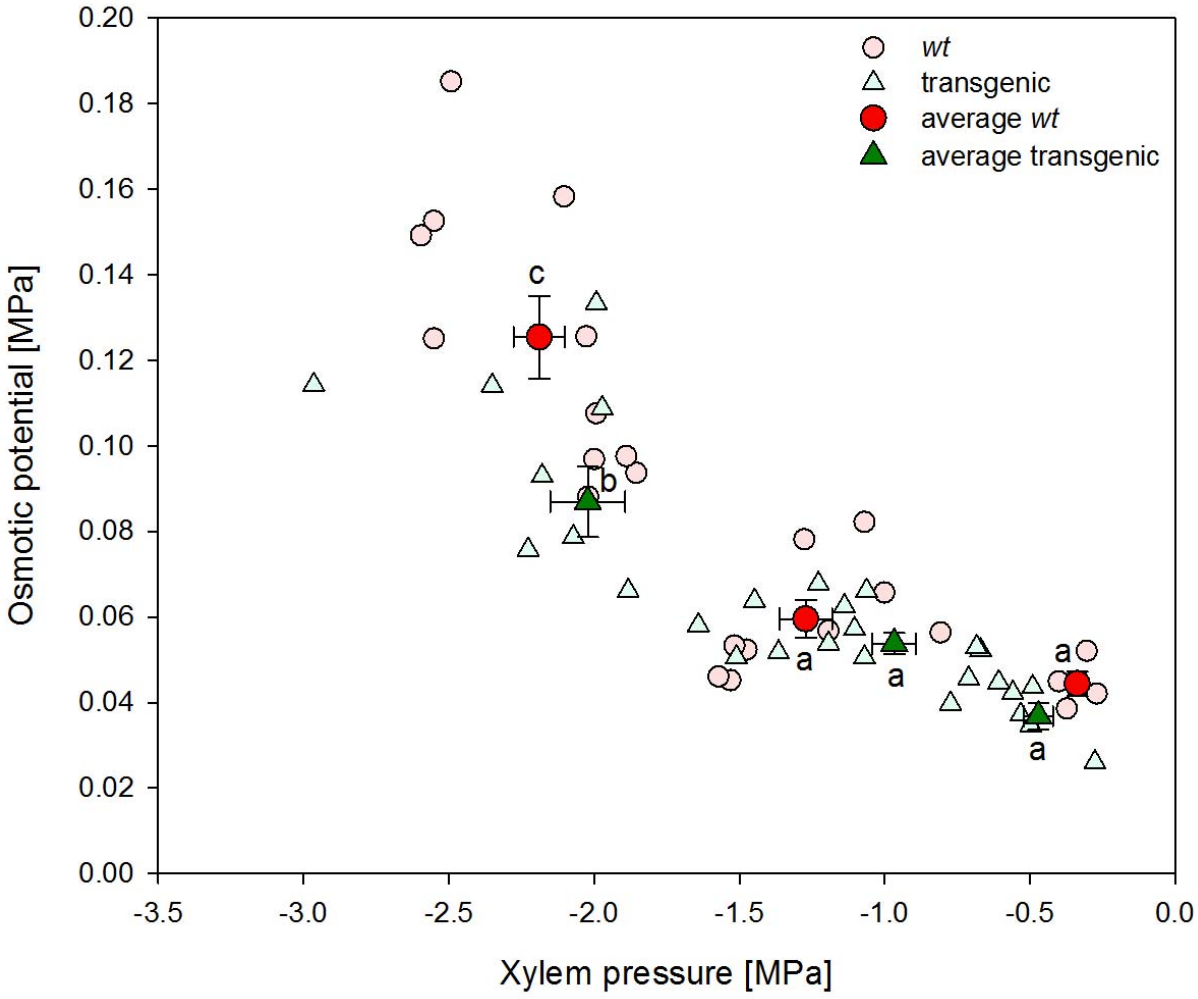

Figure 4

Changes in osmotic potential (sugar + ion) of xylem sap collected from functional vessels (*wt*, lightly colored circles; transgenic, lightly colored triangles) under different levels of xylem pressure (balancing pressure). Dark circles (*wt*) and dark triangles (transgenic) represent average values for three groups of plants well watered, moderately ($EC50_{PLC}$<50%) and severely ($EC50_{PLC}$>50%) stressed plants. The one way Anova test suggests significant differences between treatments and lines (P <0.001). Letters denote homogeneous groups based on the Fisher LSD test.

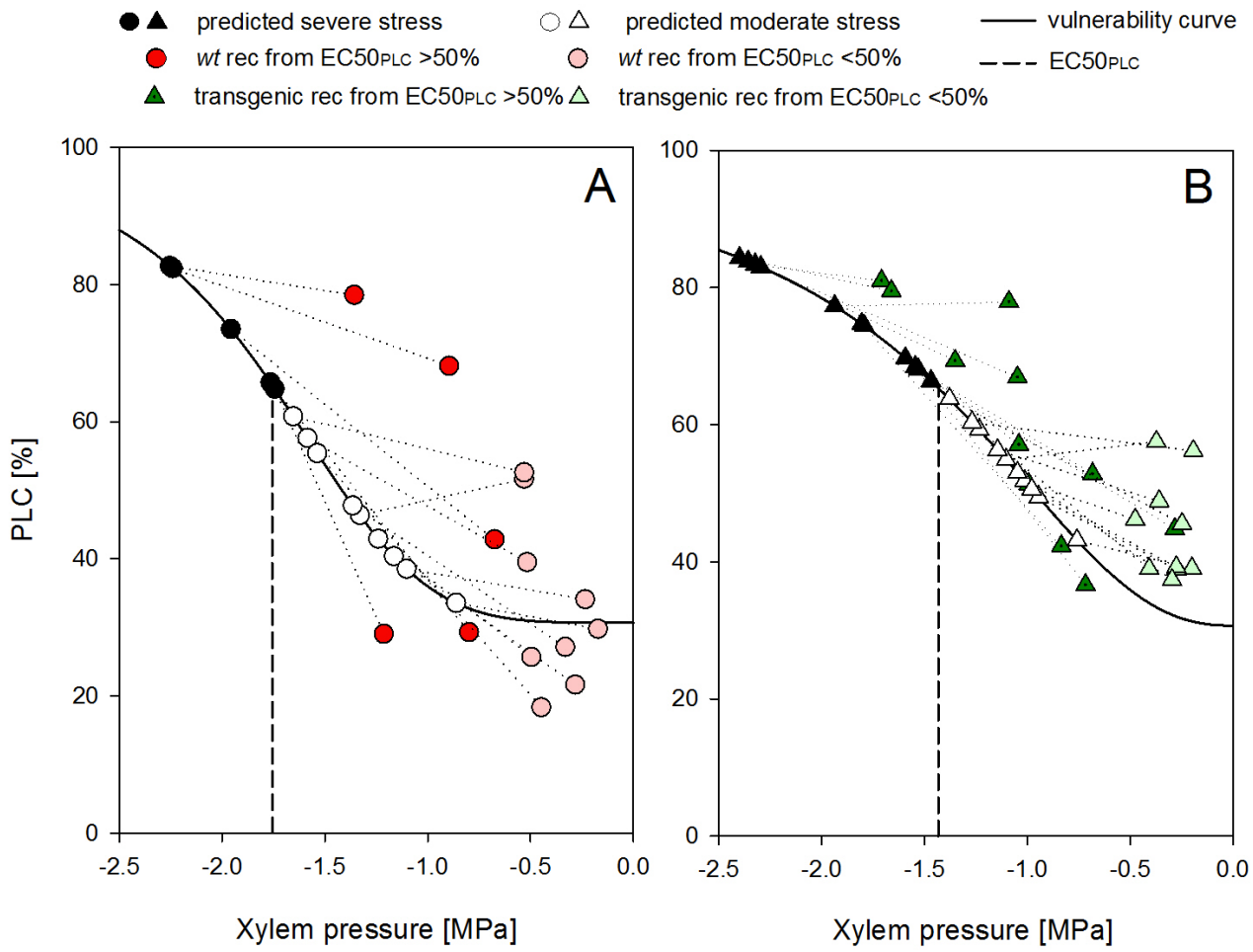

Figure 5

PLC and xylem pressure recovery from moderate ($EC50_{PLC}$ <50%) and severe ($EC50_{PLC}$ >50%) water stress levels for *wt* (A), and transgenic (B) plants occurring within 1.5 hours following re-watering. Black- and white-filled symbols represent the predicted values of PLC for severely and moderately stressed plants, respectively, and were calculated based on measured xylem pressure and the parameters of vulnerability curves. Red (*wt*) and green (transgenic) circles represent plants recovering within 1.5 hours from severe water stress, light red (*wt*) and light green (transgenic) circles show recovery from moderate stress. Dashed lines indicate $EC50_{PLC}$ = 50%.

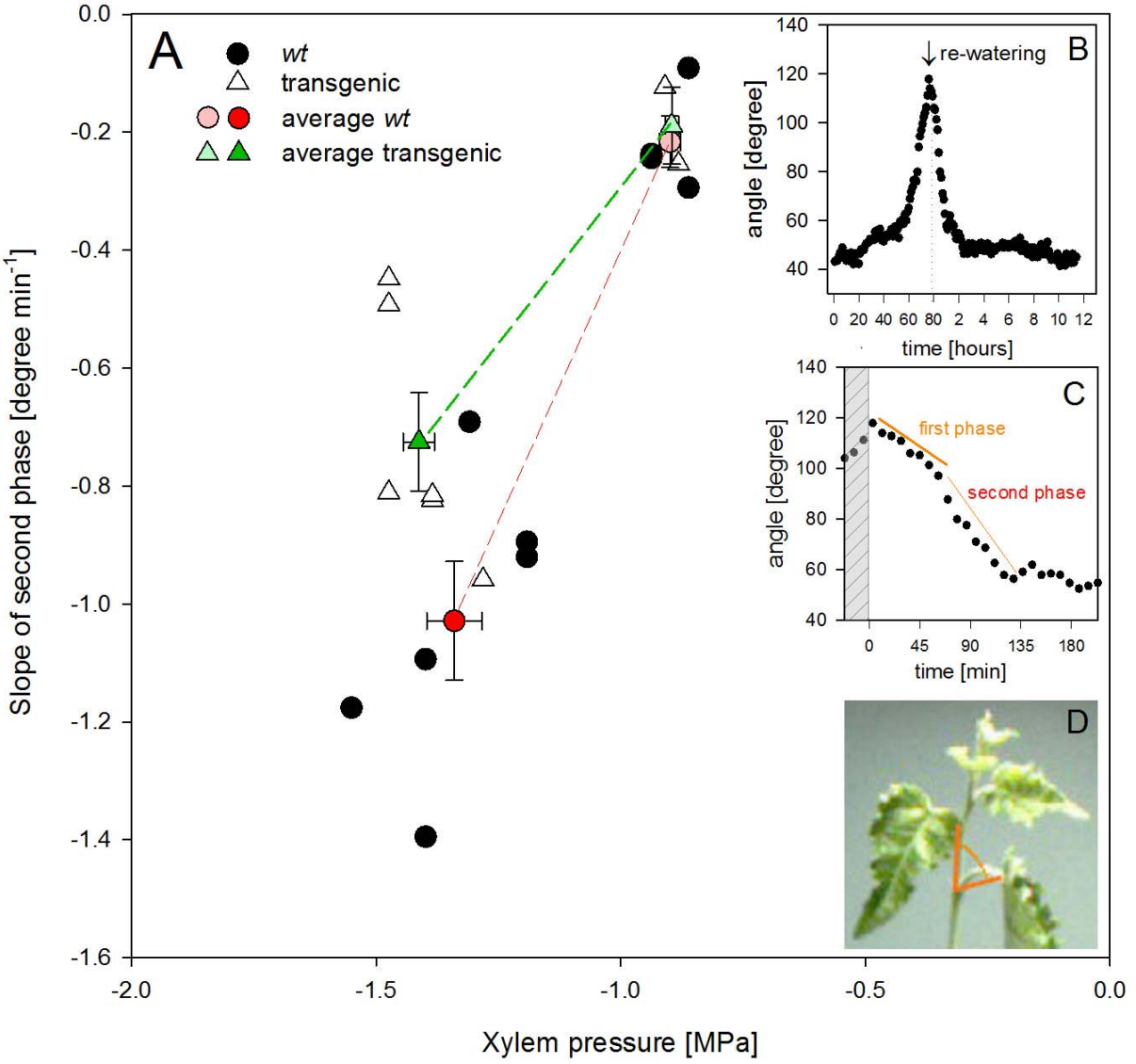

Figure 6

The rate of recovery from wilting in plants exposed to different levels of water stress expressed in degrees per minute change of the angle between stem and line connecting petiole attachment to the stem and leaf blade base (A). Data were collected from eight videos with transgenic and wt plant in each video. Statistical analysis revealed a significant difference in the slope during the second phase (fast phase) between wt and transgenic plants (wt =1.029; transgenic = -0.725; t-test; t = -2.317 df = 10, $p<0.05$). Inserts: (B) Typical changes in the angle measured during gradually increasing water stress (hours) and during recovery after re-watering (minutes). The dotted line indicates the time of re-watering. (C) The temporal dynamic of recovery is composed

of two phases—a slow phase and a fast phase. (D) Visualization of the angle between stem and line connecting petiole attachment to the stem and leaf blade base. The angle was measured every hour under increasing water stress and every 6 minutes during recovery.

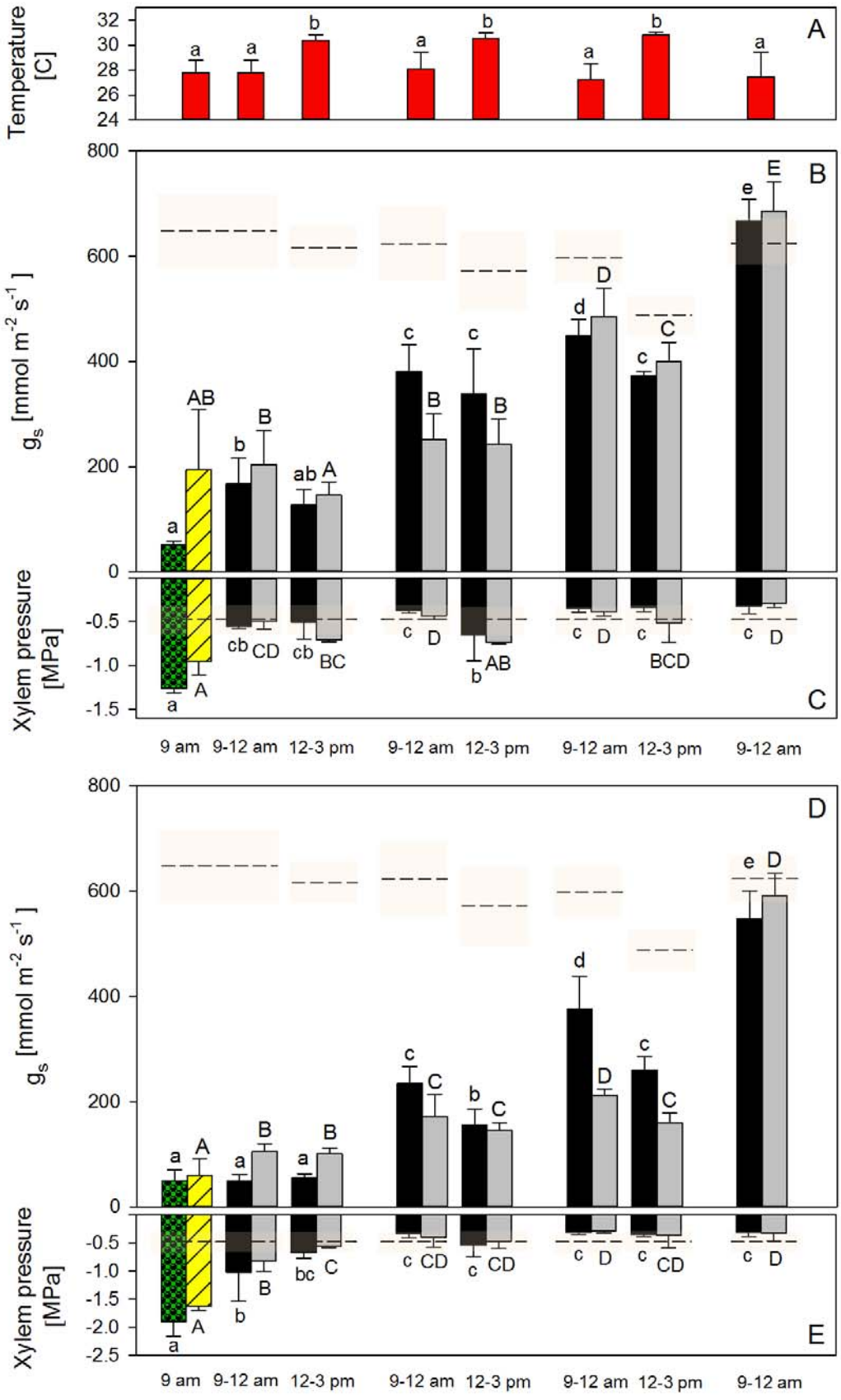
Temperature [C]
A
B
C
D
E
g_s [mmol m^{-2} s^{-1}]
Xylem pressure [MPa]
9 am
9-12 am
12-3 pm
9-12 am
12-3 pm
9-12 am
12-3 pm
9-12 am

Figure 7

Temporal dynamics of the recovery of stomatal conductance (g_s) and xylem pressure in plants recovering from moderate (B-C) and severe (D-E) water stress for *wt* (black bars) and transgenic (grey bars) lines. Measurements were conducted over four consecutive days in a greenhouse conditions. (A) provides mean values of greenhouse temperatures.

Stressed plants were re-watered the first day of the experiment a few minutes after 9 am, the time when xylem pressure and g_s values were measured [*wt*, netted pattern (green) bars; transgenic, cross pattern (yellow) bars]. Dashed lines show g_s and xylem pressure for both *wt* and transgenic well-watered controls plants [there was no difference between *wt* and PIP1 down-regulated controls for both g_s and xylem pressure; dashed lines are mean values ± SD (shaded areas)]. One way Anova test suggests significant differences between morning and afternoon greenhouse temperatures ($p< 0.001$), g_s ($p< 0.001$) and xylem pressure ($p< 0.001$) in plants recovering from moderate and severe stresses. Letters denote homogeneous groups based on the Fisher LSD method (lower-case letter, *wt*; upper-case letter, transgenic lines). Bars are mean values and error bars represent SD.

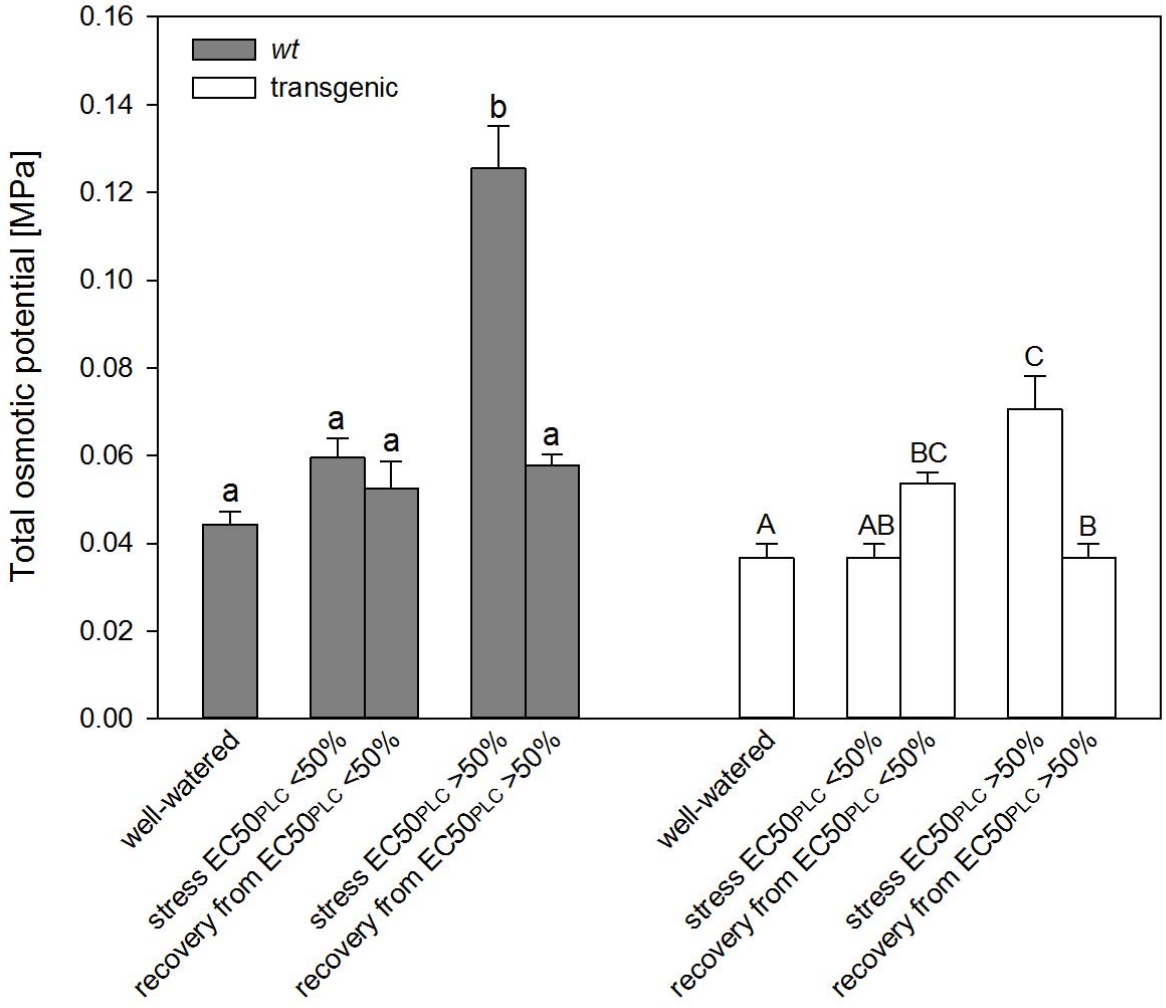

Figure 8

Total osmotic potential collected from stem xylem sap of plants recovering from moderate ($EC50_{PLC}$ < 50%) and severe ($EC50_{PLC}$ > 50%) water stress. A one way Anova test suggests significant differences between treatments in both *wt* and transgenic plants ($p < 0.001$). Letters denote homogeneous groups based on the Fisher LSD method (lower-case letter, *wt*; upper-case letter, transgenic lines). Data are mean values and bars are SD.