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

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1 **PIP1s contribute to embolism recovery**

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

17

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32 **Abstract**

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

48

49

50 **Introduction**

51 Long-distance water transport in vascular plants occurs in a conduit network of nonliving cells
52 connecting roots to leaves (Sperry, 2003). Often under drought conditions, the water column
53 within the lumen of xylem vessels or tracheids can be subjected to tensions that result in cavitation
54 and the subsequent formation of embolisms (Holbrook and Zwieniecki, 2008). This hydraulic
55 failure within the xylem network can cause tissue damage, loss of plant productivity and,
56 ultimately, plant death (Tyree and Sperry, 1989; Sperry et al., 1998; Zwieniecki and Holbrook,
57 2009). Plants have evolved several strategies to prevent and/or mitigate the effects of hydraulic
58 failure due to embolism and to restore xylem transport capacity once embolism occurs (Stiller and
59 Sperry, 2002; Nardini et al., 2011; Secchi and Zwieniecki, 2012). These strategies include
60 passive, often long-term, responses like the growth of new vessels/tracheids or dieback followed
61 by the growth of new shoots (shrubs) or active, often fast, responses that result in the restoration of
62 hydraulic failure by the formation of root pressure (Cochard et al., 1994; Ewers et al., 1997; Yang
63 et al., 2012) or stem activity (embolism removal), (Salleo et al., 2004; Nardini et al., 2011;
64 Brodersen and McElrone, 2013).

65
66 While embolism formation is a purely physical process related to the degree of tension in the
67 water column and to a wood's physicochemical properties (Brennen, 1995; Tyree and
68 Zimmermann, 2002), embolism removal requires that empty vessels fill with water against
69 existing energy gradients as the bulk of water in the xylem remains under tension due to
70 transpiration. Thus, recovery from embolism cannot happen spontaneously and necessitates some
71 physiological activities that promote water flow into embolized vessels (Holbrook and Zwieniecki,
72 1999; Tyree et al., 1999; Salleo et al., 2004; Zwieniecki and Holbrook, 2009; Secchi et al., 2011).
73 Visual evidence from cryo-SEM studies, MRI observations and CT-scans showed that water
74 (xylem sap) can return to empty vessels, suggesting that plants do have the ability to restore
75 functionality in the xylem (Holbrook et al., 2001; Clearwater and Goldstein, 2005; Scheenen et al.,
76 2007). Brodersen and colleagues showed that water droplets preferentially form on the vessel
77 walls adjacent to parenchyma cells and that these droplets grow until the lumen completely refills
78 (Brodersen et al., 2010). In addition, scientific support for the existence of embolism/refilling
79 cycles in intact stems of *Acer rubrum* are provided using magnetic resonance imaging (Zwieniecki

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

83
84 Processes related to water transport across the cellular membrane involve plasma intrinsic protein
85 (PIP- aquaporins) moderators and thus the role of PIPs must be considered when contemplating
86 how plants recover from embolism formation. Plant aquaporins show a great diversity and are
87 classified into five major homologous groups that reflect specific subcellular localizations (Prado
88 and Maurel, 2013). Among different aquaporin gene families [NIP, TIP, XIP, SIP, PIP;
89 (Danielson and Johanson, 2008)], the PIPs represent the largest number of members and can be
90 further divided into two subfamilies, PIP1 and PIP2. There is a large body of evidence that
91 aquaporins from the PIP2 subfamily contribute to water transport. The generation of data has been
92 multidisciplinary and involved the use of chemical blockers, the down and up regulation of genes
93 in plants and the expression of these proteins in oocytes (Hukin et al., 2002; Postaire et al., 2010;
94 Shatil-Cohen et al., 2011). Expression levels of several PIP and TIP members change following
95 the dynamic of increasing water stress and recovery in many woody plants including walnut,
96 poplar and grapevine (Sakr et al., 2003; Secchi et al., 2011; Perrone et al., 2012; Laur and Hacke,
97 2013; Pou et al., 2013). Further, an increase in the expression of PIP2.1 and PIP2.2 genes was
98 observed in vessel-associated parenchyma cells in walnuts at the same time that recovery from
99 embolism was taking place (Sakr et al., 2003). The role of genes from the PIP1 subfamily in tree
100 responses to water stress is less well understood. PIP1s were shown to have little to no water
101 channel activity when expressed in oocytes on their own. However, co-expression of PIP1.1
102 proteins with an isoform from the PIP2 subfamily led to higher membrane permeability than that
103 observed with the expression of a single PIP2 protein (Fetter et al., 2004; Secchi and Zwieniecki,
104 2010). With respect to their role in mediating water stress, it was shown that the expression level
105 of several PIP1 genes in poplar changed significantly during the onset of stress, during recovery,
106 during the formation of embolisms following water stress, and under no stress conditions but with
107 induced embolism, while the expression of PIP2 genes remained mostly unresponsive (Secchi and
108 Zwieniecki, 2010; Secchi et al., 2011; Secchi and Zwieniecki, 2011).

109

110 Despite significant effort invested in elucidating the contribution of aquaporins to the regulation of
111 xylem hydraulic capacity throughout the progression of drought and recovery from water stress,
112 evidence of their active role *in vivo* is only partially confirmed. Genetic approaches provide a
113 reliable and effective strategy for determining the physiological function of aquaporin genes in
114 plant water relations. However, most studies thus far have been conducted on herbaceous plants
115 (Kaldenhoff et al., 1998; Postaire et al., 2010). For example, *Arabidopsis thaliana* plants
116 expressing PIP antisense genes exhibit an impaired ability to recover from water stress (Martre et
117 al., 2002) and knock-out mutants exhibit reduced leaf hydraulic conductivity (Da Ines et al.,
118 2010). NtAQP1 down-regulated tobacco plants show reduced root hydraulic conductivity and
119 lower water stress resistance (Siefritz et al., 2002). RNA technology, although not often used for
120 woody plants, has been adapted for grapevine (Perrone et al., 2012) and for *Eucalyptus* trees
121 (Tsuchihira et al., 2010); in both cases, analysis focused on over-expressing specific isoforms of
122 aquaporin genes. The PIP2;4 root-specific aquaporin enhanced water transport in transformed
123 *Vitis* plants under well-watered conditions but not under water stress (Perrone et al., 2012), while
124 *Eucalyptus* hybrid clones over-expressing RsPIP1;1 and RsPIP2;1 did not display any increase in
125 drought tolerance (Tsuchihira et al., 2010). Up till now, no research on the recovery from
126 embolism formation in woody plants with impaired aquaporin expression has been conducted.

127
128 In the presented study, we used poplar transgenic plants characterized by a strong down-regulation
129 of PIP1 genes to test the role of this aquaporin subfamily in the plant response to water stress and
130 subsequent recovery from stress. While transformed poplars did not show morphologically
131 different phenotypes when compared to wild-type plants, they were found to be more sensitive to
132 imposed water stress resulting in increased vulnerability to embolism formation and the loss of
133 stomatal conductance. We also noted a reduced capacity of transformed plants to restore xylem
134 water transport.

135
136 **Results**

137
138 *Physiological changes in response to water stress*

139 *Populus tremula x alba* transformed trees were previously generated using a reverse genetic
140 approach (RNAi) aimed at suppressing more than one gene belonging to the poplar PIP1
141 subfamily (Secchi and Zwieniecki, 2013). Silencing the entire sub-family was preferred to
142 silencing particular isoforms in order to avoid the potential for compensation of expression within
143 that same gene group. To estimate levels of PIP1 subfamily down-regulation in the stems of five
144 selected transgenic lines, RT-PCR analyses were performed. The expression of PIP1 genes was
145 strongly reduced in all lines examined when compared to the wild-type (*wt*), decreasing by 91-
146 94% (Figure 1, ANOVA $p < 0.001$). Since there were no significant differences in PIP1 expression
147 level among lines (Figure 1), we pooled all lines into a single transformed group in order to
148 increase the sample size in subsequent physiological experiments. Once pooled, analysis yielded a
149 93% reduction in expression for PIP1 subfamily gene compared to wild-type (*wt*), (Figure 2, *t*-test;
150 $p < 0.001$). Additionally, PIP1.1 and PIP1.3 genes [two highest expressed genes in stems from
151 PIP1 subfamily (Secchi et al., 2009) and the most responsive genes to drought and embolism
152 formation (Secchi and Zwieniecki, 2010)] showed reduced expression levels in the combined
153 transgenic stems confirming the successful down regulation of multiple isoforms belonging to the
154 same subfamily. The expression of the other genes belonging to PIP1 subfamily was also
155 monitored. All PIP1 genes were strongly down-regulated and resulted to be significant different
156 from their expression in stems of *wt* plants (Figure 2 and Supplemental Online Material Figure
157 S1A). To test the possible compensatory response of the PIP2 gene subfamily members in
158 response to PIP1 down-regulation, the transcript levels of seven out of eight genes belonging to
159 PIP2 subfamily were measured (PIP2.8 was analyzed but its expression was not detected in the
160 stem tissue). In general, their gene expressions were not significantly different from *wt* plants
161 suggesting a lack of PIP2 compensatory response (Figure 2 and Online Material Figure S1B).

162
163 Both *wt* and PIP1 down-regulated poplars showed similar levels of native embolism in well-
164 watered plants averaging around 30.7% and 37.3%, respectively (Figure 3A). An increase in
165 water stress resulted in additional losses of stem hydraulic conductivity plateauing above 80%,
166 below xylem pressures (P_{xylem}) of -2.3 MPa in both groups. The relationship between the loss of
167 hydraulic conductivity and xylem pressure was fitted using four-parameter logistic curves (dose
168 response curves – see methods). The 50% loss of effective stem conductivity (not including

169 native embolism) described by the $EC50_{PLC}$ parameter of the curve (half maximal '*effective*
170 *concentration*' - in our case '*effective xylem pressure*') occurred at -1.76 MPa (SE = 0.0642, $t =$
171 27.3493, $p < 0.0001$) for *wt* plants and at -1.43 MPa (SE = 0.0816, $t = 17.54$, $p < 0.0001$) for
172 transgenic plants. The $EC50_{PLC}$ parameter was significantly different between the two groups [Z-
173 test; $t = 3.120$, $df = 47$, $p < 0.0025$; (Paternoster et al., 1998)], indicating that the stems of
174 transgenic lines were more vulnerable to embolism than the stems of *wt*. For the purpose of
175 clarity we will later refer to plants as moderately stressed when xylem pressure is above $EC50_{PLC}$
176 (i.e. $PLC < 50\%$, but below xylem pressure of well watered plants) and severely stressed if xylem
177 pressure is below $EC50_{PLC}$ (i.e. $PLC > 50\%$).

178

179 Stomatal conductance (g_s) was similar for non-stressed (well watered) *wt* and PIP1 down-
180 regulated plants (~ 600 mmol $m^{-2} s^{-1}$), (Figure 3B). A decrease in g_s was observed with an
181 increase in water stress, and both groups showed full stomatal closure at xylem pressures below
182 -2.0 MPa. Changes in stomatal conductance in response to xylem pressure were fitted with the
183 'dose-response' curve. An effective drop in g_s by 50% from its observed maximum in well-
184 watered plants ($EC50_{g_s}$) was at -1.102 MPa for *wt* and at -1.316 MPa for transgenic lines. There
185 was a statistical difference between $EC50_{g_s}$ parameters of the two groups (Z-test; $t = 2.066$, $df =$
186 43, $p < 0.025$). Importantly, *wt* plants closed their stomata by 50% at approximately ~ 0.6 MPa
187 before $EC50_{PLC}$, providing a relatively wide PLC safety margin, while transgenic plants closed
188 stomata at only ~ 0.1 MPa ahead of $EC50_{PLC}$, providing a very narrow safety margin. However,
189 both groups completely closed their stomata before the maximum loss of stem conductance
190 occurred.

191

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

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

202

203 *Physiological changes upon recovery from induced water stress*

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

216

217 Direct observation of plant recovery from stress using time-lapse imaging showed that turgor
218 recovery in leaves could be characterized by two phases; (1) a slow phase – that lasted for 37-40
219 minutes – and was characterized by a slow steady decrease in the angle between the petiole and
220 the stem and (2) a fast phase – that lasted more than 40 minutes – characterized by a fast change in
221 the angle that resulted in total recovery of initial leaf position (Figure 6). The rate of recovery in
222 the slow phase was similar in both groups of plants. The rate of recovery during the fast phase
223 was only similar in plants recovering from low stress levels above $EC_{50_{PLC}}$ (P_x was less negative
224 than $EC_{50_{PLC}}$). However, recovery from stress around $EC_{50_{PLC}}$ or lower (P_x was equal to or more
225 negative than $EC_{50_{PLC}}$) was significantly slower in transgenic plants resulting in a delay of several
226 minutes in restoration of turgor and pre-stress leaf positions (Figure 6).

227

228 The recovery of stomatal conductance did not follow the patterns of fast recovery observed in P_x
229 (minutes to a couple of hours; Figure 7), leaf turgor (Figure 6) and PLC (Figure 5). Full g_s
230 recovery did not occur until after 4 days of full irrigation. The general pattern of g_s recovery was
231 also different in plants recovering from moderate stress and severe stress between *wt* and
232 transgenic plants. Moderate stress only forced full stomata closure in *wt* plants while transgenic
233 stomata remained partially open (Figure 7; and compare Figure 3B). Wild-type moderately
234 stressed plants showed signs of some g_s recovery immediately after re-watering (first day - Figure
235 7 B and C). The recovery was not observed in transgenic plants despite their tendency to have
236 higher initial (under stress) g_s . The recovery of g_s continued in *wt* plants during the second day,
237 reaching the g_s of control, non-stressed plants during the third day. Full recovery occurred four
238 days after the return of irrigation. Transgenic plants did not show significant signs of g_s recovery
239 in the second day but recovery was similar to *wt* plants in third and fourth day. Severe stress
240 (below $EC_{50_{PLC}}$) forced full stomatal closure in both *wt* and transgenic plants. Recovery from
241 severe water stress did not start during the first day for either plants group, but later showed a
242 similar pattern to that observed in recovery from moderate stress. Again, the start of the partial
243 recovery of g_s in transgenic lines was delayed one day, when compared to *wt* (Figure 7 D-E).
244 Differences of g_s values between mornings and afternoons were related to variation in greenhouse
245 temperature (Figure 7A).

246
247 The osmotic potentials (sugars and ions) of sap collected from functional vessels during recovery
248 from stress are accounted for by ion concentration with little contribution from sugar content
249 (Supplemental Figure 3). Wild-type plants recovering from moderate and severe stress showed
250 osmotic potential values similar to those measured in well-watered plants (Figure 8, grey bars),
251 with the exception of higher osmotic values found under severe stress. Liquid collected from
252 moderately and severely stressed transgenic plants had lower osmoticum concentrations than
253 control plants (Figure 8, white bars), suggesting that these plants may be impaired in their
254 utilization of ions in their response to stress.

255

256 **Discussion**

257

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

267

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

285

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

288 PLC at less negative xylem pressure (-1.4 MPa vs -1.7 MPa for transgenic and *wt* plants,
289 respectively), indicating that the presence of PIP1 genes might be beneficial to plant
290 vulnerability resistance (Figure 3A). This increased vulnerability to embolism in transgenic
291 plants was associated with their reduced capacity to control stomatal conductance during stress
292 development, suggesting a different content of abscissic acid (ABA) in the tissues and
293 consequently a delay of stomata closure in response to drought stress. In transgenic plants, 50%
294 of stomatal shutdown occurred at -1.3MPa and in *wt* plants, at -1.1 MPa, with approximately
295 40% of maximum g_s at $EC_{50_{PLC}}$ for transgenic plants and less than 10% of maximum g_s at
296 $EC_{50_{PLC}}$ for *wt* plants (Figure 3B). Since stomatal closure is one of the mechanisms that plants
297 can adopt in order to limit water loss and control stress level, the behavior assumed by transgenic
298 plants suggests that they were less likely to control transpiration rates to protect xylem from
299 embolism formation. In other words, the down regulation of PIP1 gene expression resulted in a
300 significant reduction of the xylem vulnerability safety margin (Sperry and Ikeda, 1997; Choat et
301 al., 2012; Johnson et al., 2012).

302
303 As embolism formation is mostly a physical process it might be hard to imagine how PIP1 genes
304 are able to influence a plant's vulnerability curve without also affecting morphology or xylem
305 anatomy. However, previous studies have shown a positive correlation between PIP1 expression
306 and stress conditions, as well as the up-regulation of PIP1 expression in response to embolism
307 formation even in the absence of water stress (Secchi and Zwieniecki, 2010). Thus, in
308 combination with results presented here we can propose that the processes of embolism
309 formation and refilling are not separated in time but happen simultaneously with respective rates
310 that are functionally linked to plant stress level. Embolism formation rates are expected to
311 increase with increases in stress while embolism refilling rate are expected to decrease with
312 stress. To rephrase, the current level of PLC at any given moment is a result of embolisms
313 formed minus refilling. As the down-regulation of PIP1 genes negatively affects refilling rate,
314 the result of the competition between the two processes shifts transgenic plants towards an
315 apparently higher vulnerability to embolism formation while this is in fact a result of reduced
316 capacity to refill. Thus, PIP1s do not directly influence cavitation thresholds but do reduce
317 refilling rates.

318

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

339

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

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

355
356 The function of PIP1 genes in promoting tolerance to water stress has been studied through
357 reverse genetic approaches that over or under express aquaporin genes (Martre et al., 2002; Cui
358 et al., 2008; Kaldenhoff et al., 2008; Zhang et al., 2008; Postaire et al., 2010; Sreedharan et al.,
359 2013). Conclusions have been made with antisense tobacco plants; under well-watered
360 conditions the NtAQP1 aquaporin did not seem to be important for water uptake or management.
361 However, in a water limited environment NtAQP1 antisense plants were not able to maintain
362 turgor and appeared to be less drought tolerant when compared with wild-type tobacco plants
363 (Siefritz et al., 2002). When subjected to drought, tobacco plants with overexpressed BnPIP1
364 were more tolerant to water stress, while the antisense lines with reduced mRNA levels of
365 BnPIP1 showed reduced water uptake and were more sensitive to water deficiency (Yu et al.,
366 2005). Results presented here suggest that the major effect of the PIP1 gene subfamily on the
367 stress physiology of woody plants is directly linked to plant management of apparent
368 vulnerability to embolism (Secchi and Zwieniecki, 2010; Secchi and Zwieniecki, 2012).
369 Specifically, we propose here that the observed increased vulnerability to embolism formation
370 and the significant delay in recovery of hydraulic capacity in transgenic plants indicates that a
371 loss of PIP1 gene expression reduces the rate of refilling and effectively shifts the balance
372 between the rates of embolism formation and refilling such that embolism became a dominate
373 process at lower tensions. Apparent increased of vulnerability without obvious phenotypic
374 differences between *wt* and transgenic plants underlines the role of physiology in the
375 maintenance of xylem hydraulic capacity and suggests that continuous competition between the
376 processes of embolism formation and removal might be mediated by PIP1 activity (Zwieniecki
377 and Holbrook, 2009; Zwieniecki et al., 2013).

378

379 **Material and Methods**

380 *Plant materials and experimental design*

381 Wild-type (*wt*) and transgenic hybrid white poplars (*Populus tremula* x *Populus alba*, INRA-
382 France clone 717-1B4) were used for the study. Down-regulated PIP1 transgenic plants were
383 previously generated and described in Secchi and Zwieniecki (2013). Poplars were grown in a
384 greenhouse with the following ambient conditions: temperature maintained in the range of 25°C to
385 32°C and natural daylight supplemented with light from metal halogen lamps to maintain a
386 minimum of 500–600 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ during 12/12-h light/dark cycle. Plants were
387 approximately 1.5 m tall at the onset of the experiments.

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

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

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

413
414 *Expression of aquaporin genes in transgenic poplars*

415 Total RNA was isolated from *wt* and transgenic stems according to the protocol of Chang, (Chang
416 et al., 1993). First strand cDNA was synthesized from total RNA treated with DNase I
417 (Fermentas) using oligo(dT)₁₂₋₁₈ as primers (Fermentas) and SuperScript II Reverse Transcriptase
418 (Invitrogen). The sequences of primers used for Real time PCR analysis are listed in the Table S2.
419 Primers were tested on cDNA of hybrid poplar through PCR with RED Taq DNA Polymerase
420 (Sigma) according to the manufacturer's instructions. The transcript abundance of each gene was
421 quantified with SYBR Green JumpStart Taq Ready Mix (Sigma) on an Eco Real-Time PCR
422 System (Illumina, San Diego, USA). Thermo-cycler conditions for all real-time analyses were:
423 95°C for 5 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30s.
424 Data were analyzed using Eco software (Illumina), and the expression values were normalized to
425 the geometric mean of two housekeeping genes (ubiquitin and actin). These genes were found to
426 have, for the same poplar species, the highest amplification efficiency and most stable expression
427 across different tissue (Carraro et al., 2012). Real-time PCR was carried out using three
428 biological replicates per transformed line. Two technical replicates were performed for each of
429 the three biological replicates.

430
431 *Measurements of xylem pressure and stem hydraulic conductivity*

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

437 Stem hydraulic conductivity was measured using a standard approach previously described
438 (Secchi and Zwieniecki, 2010). Briefly, 1.5m long shoot was cut under water. Within few
439 minutes this initial cut was followed with cutting a set of set of 3 stem segments. Segments were
440 excised under water, approximately 20-30 cm from the initial cut (distance longer than 2x length
441 of vessels in studied poplar). Each segment was approximately ~4 cm long. The initial
442 hydraulic conductance (k_i) of each stem segment was measured gravimetrically by determining
443 the flow rate of filtered 10 mM KCl solution. A water source was located on a balance (Sartorius
444 ± 0.1 mg) and connected to the stem by a plastic tube. During measurements stems were
445 submerged in a water bath with a water level ~10 cm below the level of water on the balance.
446 After a steady flow rate was reached (within a few minutes), the tube connecting the stem to the
447 balance was closed, and a bypass tube was used to push water across the segment under ~ 0.2
448 MPa of pressure for approximately 20 s to remove embolism. The chosen segment length used
449 for determining PLC was short enough to have the majority of vessels open in poplar stems
450 (vessel length is usually ~5cm), thus making removal of embolism very easy and complete
451 within few seconds. Stem conductance was then re-measured to find maximum conductance
452 (k_{max}). The percent loss of conductance (PLC) was calculated as $PLC = 100 * (k_{max} - k_i) / k_{max}$. The
453 same procedure was used in experiment 1 and 2.

454

455 *Measurements of stomatal conductance*

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

461

462 *Curve fitting*

463 Relationships between percent loss of conductance (PLC) and stomatal conductance (g_s) in
464 response to stem water pressure were fitted with a four-parameter logistic curve (dose response);
465 where: $PLC = initial_{PLC} + (maximum_{PLC} - initial_{PLC}) / (1 + (P_{xylem} / EC50_{PLC})^{Slope_{EC50_{PLC}}})$, and
466 $g_s = minimum_{gs} + (initial_{gs} - minimum_{gs}) / (1 + (P_{xylem} / EC50_{gs})^{Slope_{EC50_{gs}}})$. This function was

467 preferred over other sigmoidal shapes as it allows treating xylem pressure as a treatment (dose)
468 and allows for the fit of initial values to true preexisting conditions. $EC50_{PLC(g_s)}$ is the parameter
469 describing a 50% change in the curve between the initial value of PLC or g_s and the
470 corresponding final value at very low xylem pressures (PLC-maximum and g_s -minimum).
471 $Slope_{PLC(g_s)}$ describes the rate of change in PLC or g_s at the inclination point of the curve. In
472 order to compare *wt* and transgenic plants PLC and g_s response to xylem water pressure, we
473 compared $EC50_{PLC(g_s)}$ parameters of fitted curves using the corrected statistical Z-test for the
474 equality of regression coefficients (Paternoster et al., 1998).

475

476 *Carbohydrate and ion contents in xylem sap of functional vessels*

477 The xylem sap of functional vessels was collected from the same plants that were used to
478 determine PLC and xylem pressure (experiment 1 and 2) using the procedure previously
479 described (Secchi and Zwieniecki, 2012). Briefly, leaves were removed and the stem was
480 attached through a plastic tube to a syringe needle. The needle was threaded through a rubber
481 cork to a vacuum chamber with the needle tip placed in the 1.5 ml plastic tube. After the
482 generation of a vacuum, short pieces of stem were consecutively cut from the top allowing
483 liquid from open vessels to be sucked out of the stem and collected in the tube. Collected
484 liquid was then analyzed for sugar and ion concentrations following the procedures described
485 in detail by (Secchi and Zwieniecki, 2012). Briefly, carbohydrate content was quantified using
486 the colorimetric anthrone-sulfuric acid assay (Leyva et al., 2008). 50 μ l of xylem sap were
487 added to 150 μ l of fresh anthrone reagent, samples were mixed, kept 10 min at 4 °C and then
488 incubated 20 min at 100 °C. After heating, they were cooled for 20 min at room temperature
489 and absorbance at 620 nm was read with a microplate multiscan reader (Multiscan Thermo
490 Scientific). Total carbohydrate content was calculated as mg/ml of glucose, and from the
491 deduced molal concentration of each xylem sap solution the relative osmotic potential was
492 calculated based on the law for perfect gases: $\Pi = miRT$. Where: m = molality of the solution
493 (moles of solutes/1000g H₂O), i = a constant that accounts for ionization of the solute, for
494 glucose $i = 1$, R = the gas constant (0.00831 liter MPa mol⁻¹ K⁻¹) and T = temperature, 293.16
495 K.

496

497 Ion concentration was measured as electrical conductivity using a 5 μ l capillary fitted with
498 gold electrodes at the both ends and connected to a multi-meter (True RMS digital multimeter
499 289; Fluka Europe). Liquid samples were sucked into the capillary using a pipettor. A series
500 of potassium chloride solutions with different concentrations was used to establish a
501 calibration curve. Electrical conductivity of xylem sap was translated to the equivalent
502 concentration of potassium ions.

503

504 *Movies on drought*

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

511

512 *Supplemental data*

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

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

524

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528

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530

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687

688

689

690 **Figure legends**

691 **Figure 1**

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

697

698 **Figure 2**

699 Relative expression levels of PIP1 subfamily gene (transgenic construct), PIP1s (1-5) and PIP2s
700 (1-7) genes for pooled transgenic *P. alba x tremula* stems tested against expression level in *wt*
701 stems. Data are mean values and the error bars represent SE. Letters denote homogeneous groups
702 based on the Fisher LSD corrected for the multiple comparisons. Anova test revealed the presence
703 of significant differences for all PIP1 genes tested ($p < 0.001$), while no differences were founded
704 among all PIP2 genes tested in transgenic when compared to *wt* plants.

705

706 **Figure 3**

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

709 Data were fitted with the four-parameter logistic curves ('dose response curve', full lines for *wt*;
710 dotted/dash black lines for transgenic) in the form of: $PLC = \min PLC + (\max PLC - \min PLC) / (1 + (\Psi / EC50_{PLC})^{\text{slope}})$, where $\min PLC$ was the minimum PLC in well-watered plants,
711 $\max PLC$ was 100%, $EC50_{PLC}$ representing a 50% loss of initial functionality $[(\min PLC +$
712 $(\max PLC - \min PLC)]$ (half maximal 'effective concentration' - in our case 'effective xylem
713 pressure'), and slope the rate of PLC increase at $EC50_{PLC}$. Same function was used to fit the g_s
714 response to stem water pressure. Red circles/dash line and green triangles/dash line represent
715 $EC50_{PLC}$ for *wt* and transgenic plants, respectively; while red and green stars/full lines represent a
716 50% loss of initial g_s ($EC50_{g_s}$) for *wt* and transgenic plants, respectively. Parameters that describe
717 curves for the two population of plants are statistically different (*wt* $EC50_{PLC} = -1.756$ and
718

719 transgenic $EC50_{PLC} = -1.432$; t-test, $p < 0.0025$; wt $EC50_{g_s} = -1.102$ and transgenic $EC50_{g_s} = -$
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**

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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 \pm 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
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766 error bars represent SD.

767

768 **Figure 8**

769 Total osmotic potential collected from stem xylem sap of plants recovering from moderate
770 ($EC_{50_{PLC}} < 50\%$) and severe ($EC_{50_{PLC}} > 50\%$) water stress. A one way Anova test suggests
771 significant differences between treatments in both *wt* and transgenic plants ($p < 0.001$). Letters
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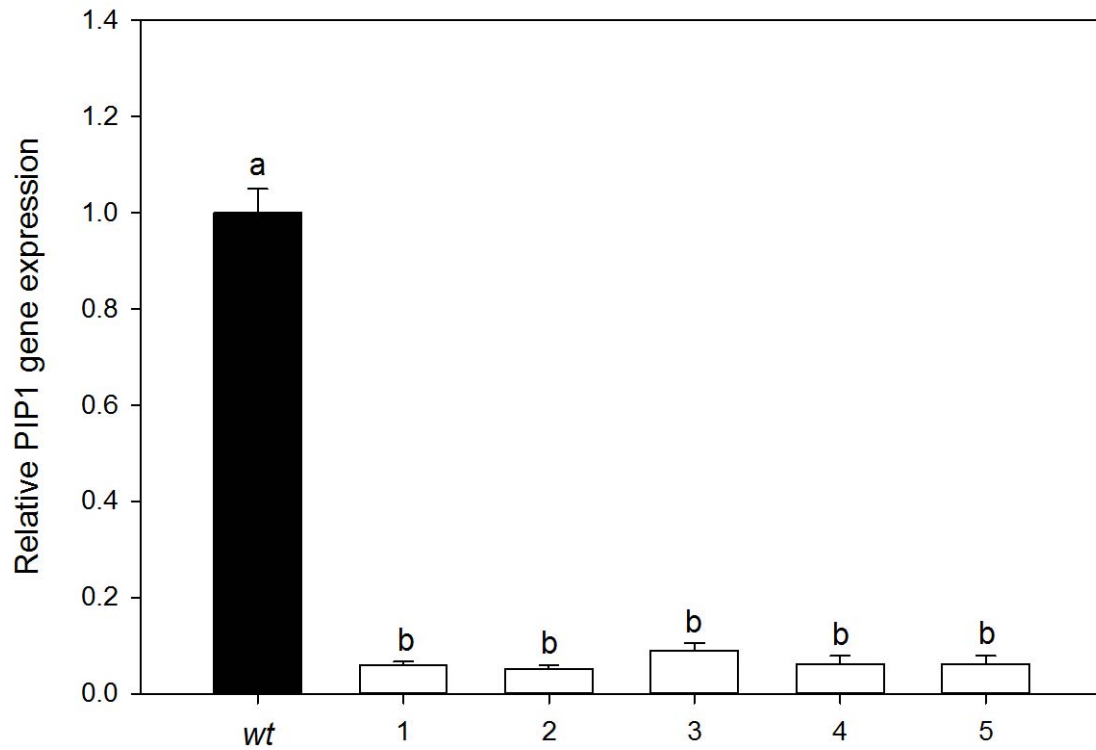


Figure 1

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).

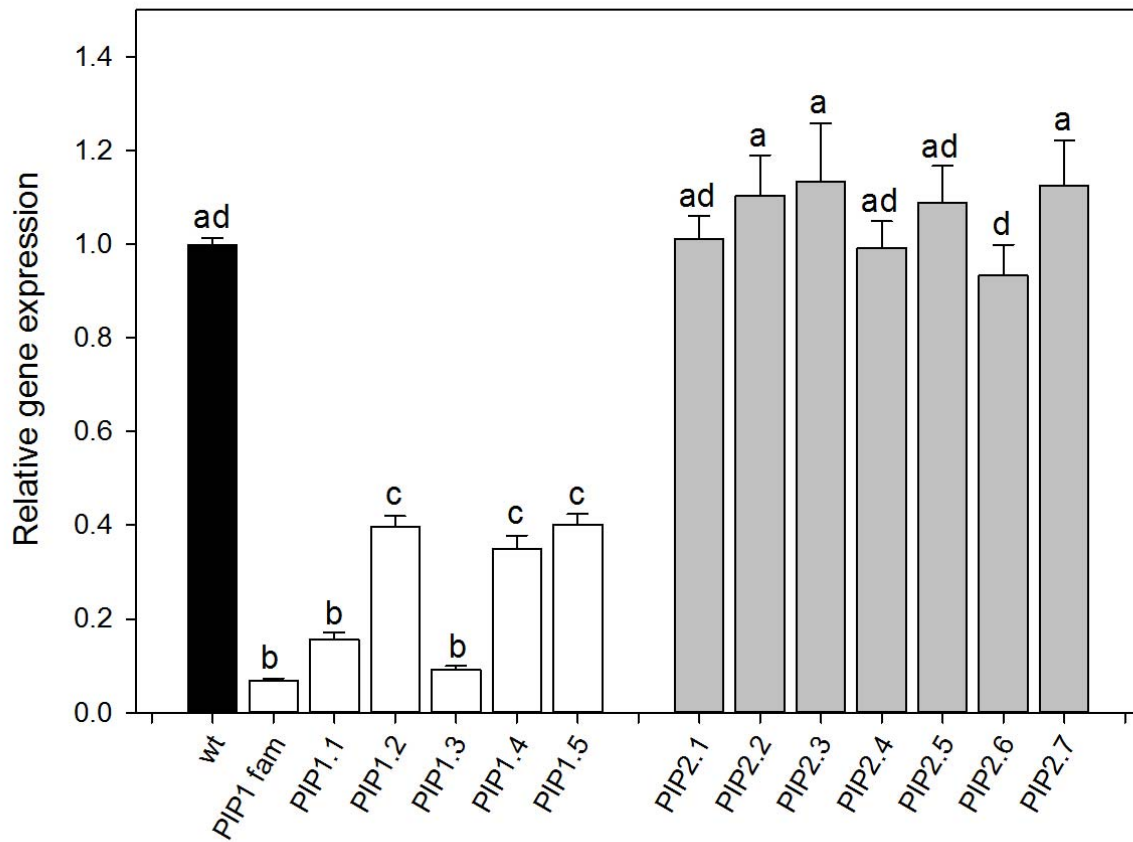


Figure 2

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.

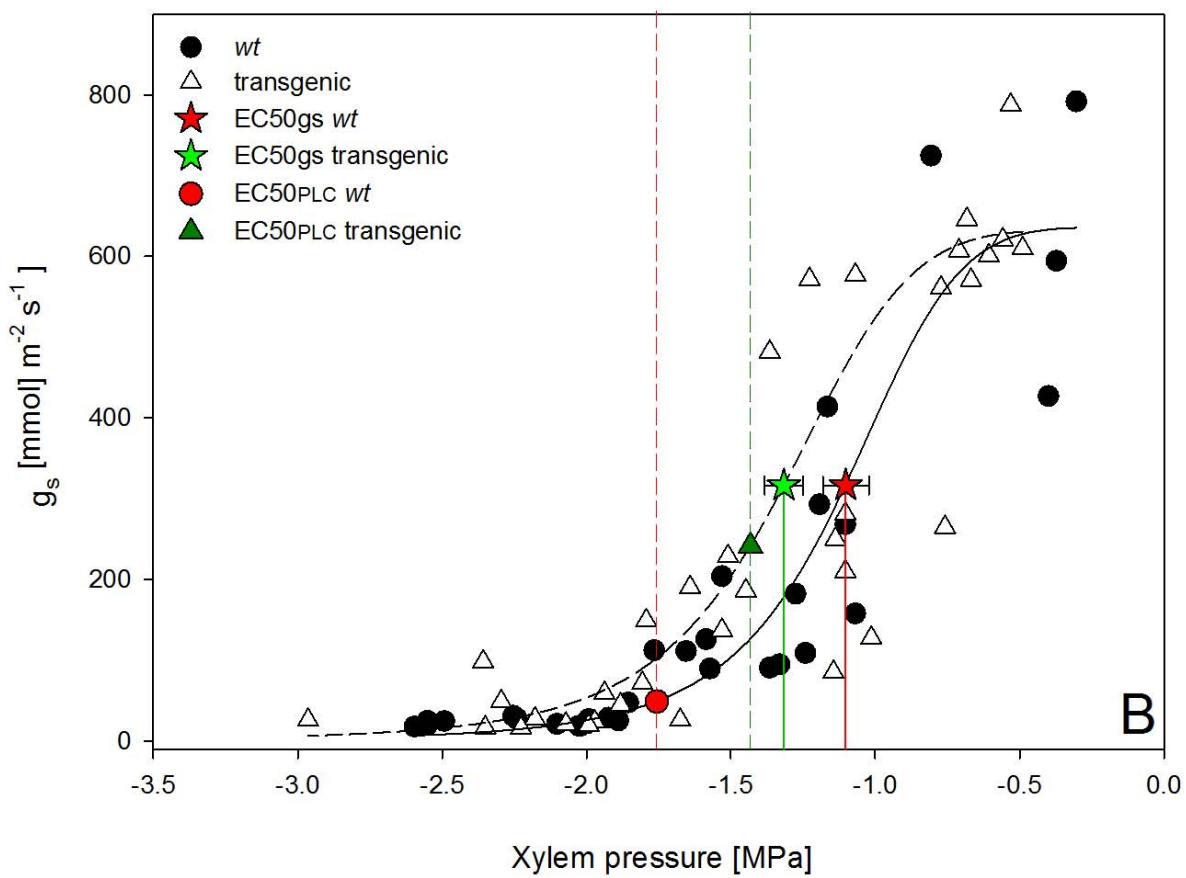
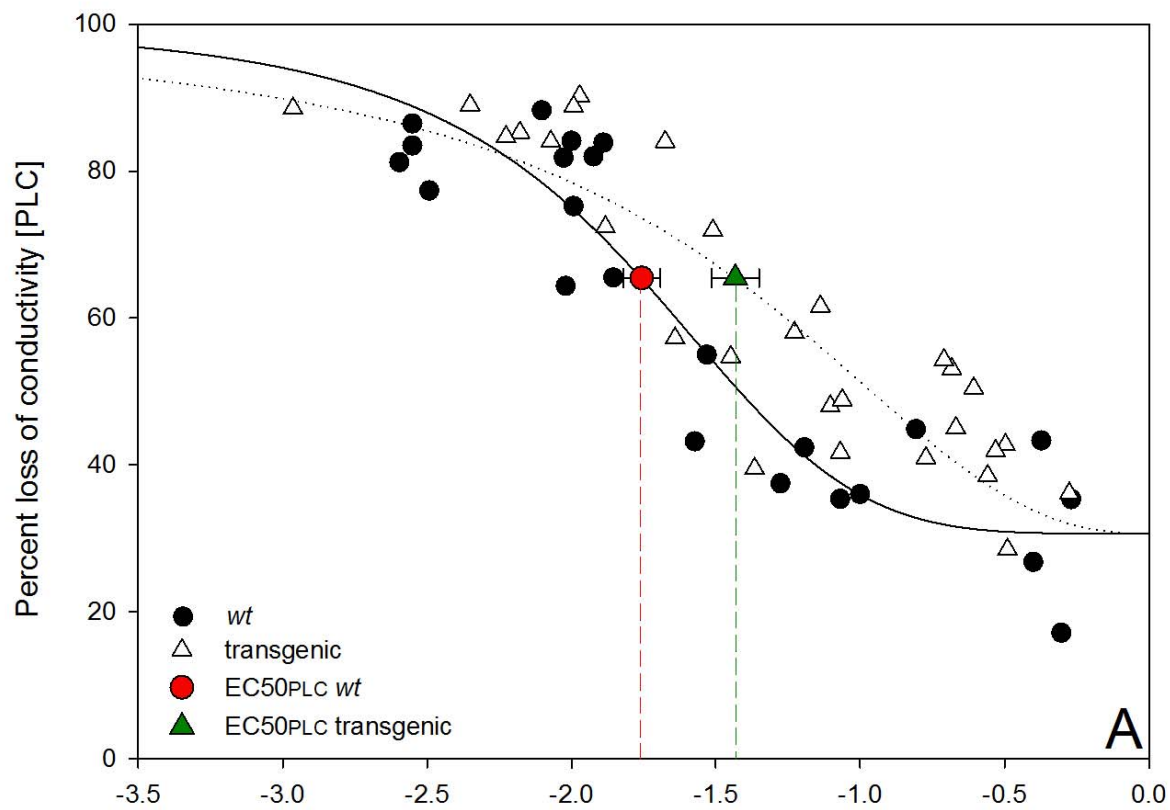


Figure 3

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: $PLC = \min PLC + (\max PLC - \min PLC) / (1 + (\Psi / EC50_{PLC})^{\text{slope}})$, where $\min PLC$ was the minimum PLC in well-watered plants, $\max PLC$ was 100%, $EC50_{PLC}$ representing a 50% loss of initial functionality [($\min PLC + (\max PLC - \min PLC)$] (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 ($EC50_{g_s}$) 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 transgenic $EC50_{PLC} = -1.432$; *t*-test, $p < 0.0025$; *wt* $EC50_{g_s} = -1.102$ and transgenic $EC50_{g_s} = -1.316$, *t*-test, $p < 0.025$).

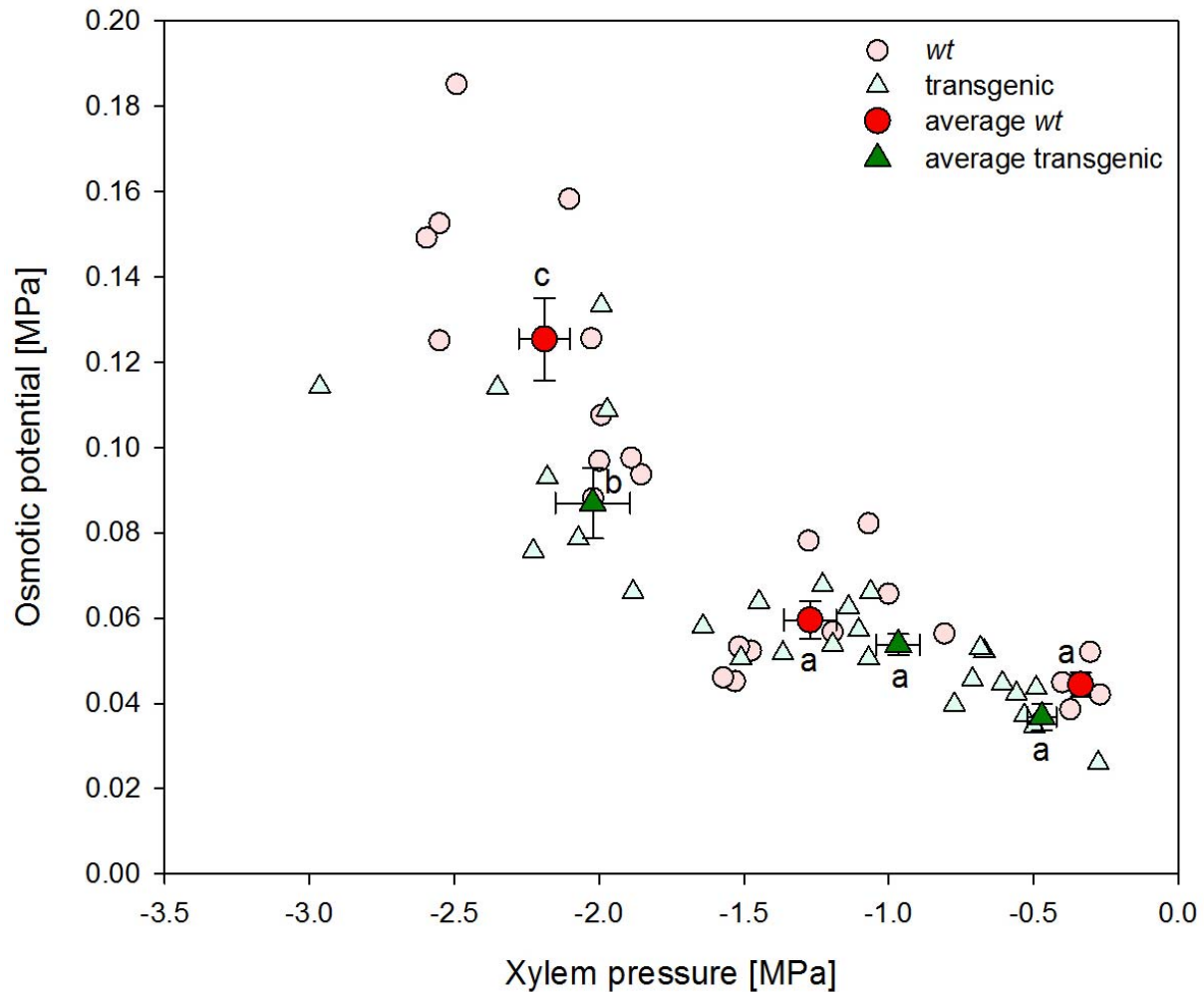


Figure 4

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 ($EC_{50_{PLC}} < 50\%$) and severely ($EC_{50_{PLC}} > 50\%$) 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.

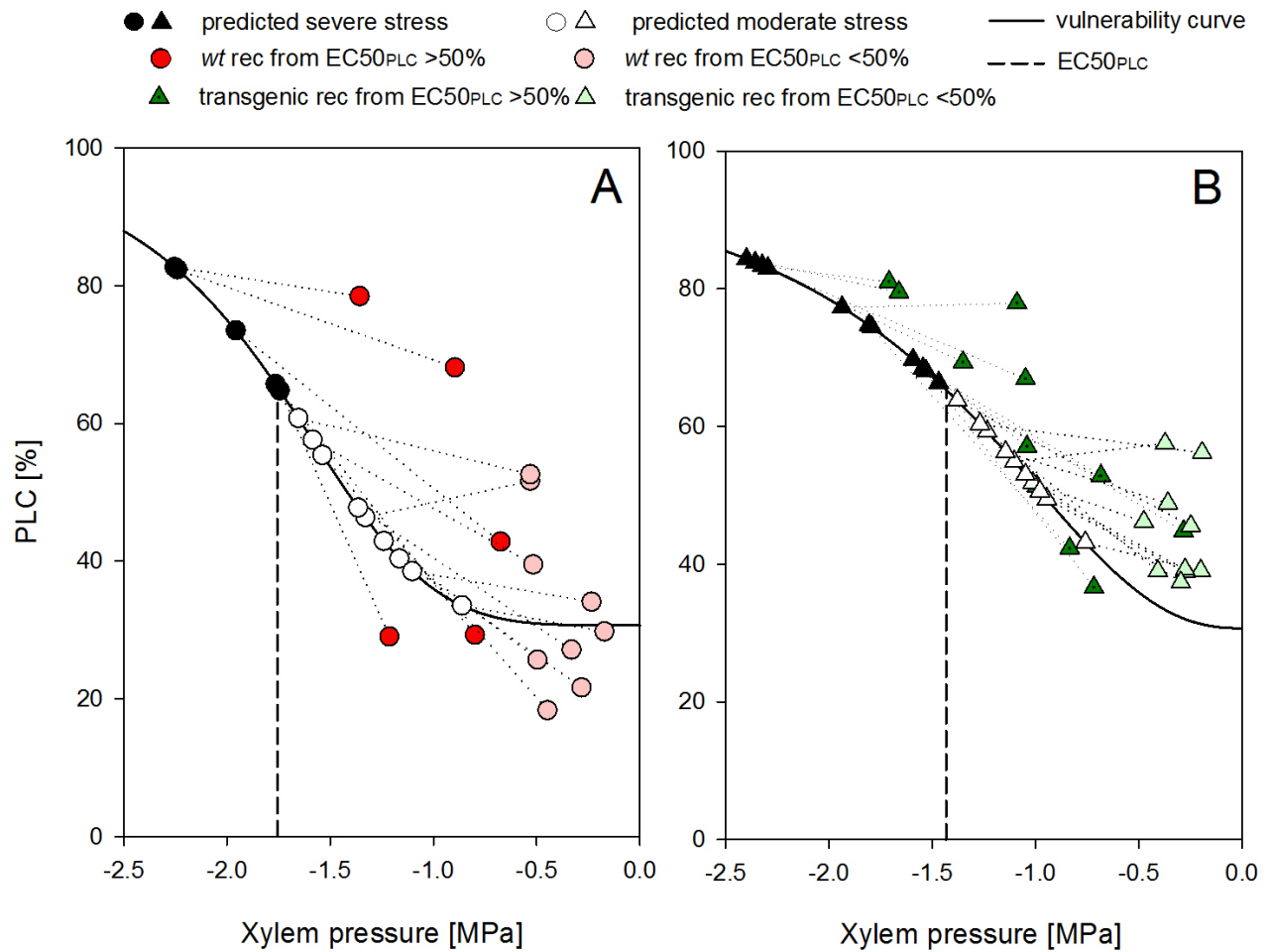


Figure 5

PLC and xylem pressure recovery from moderate ($EC_{50_{PLC}} < 50\%$) and severe ($EC_{50_{PLC}} > 50\%$) 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 $EC_{50_{PLC}} = 50\%$.

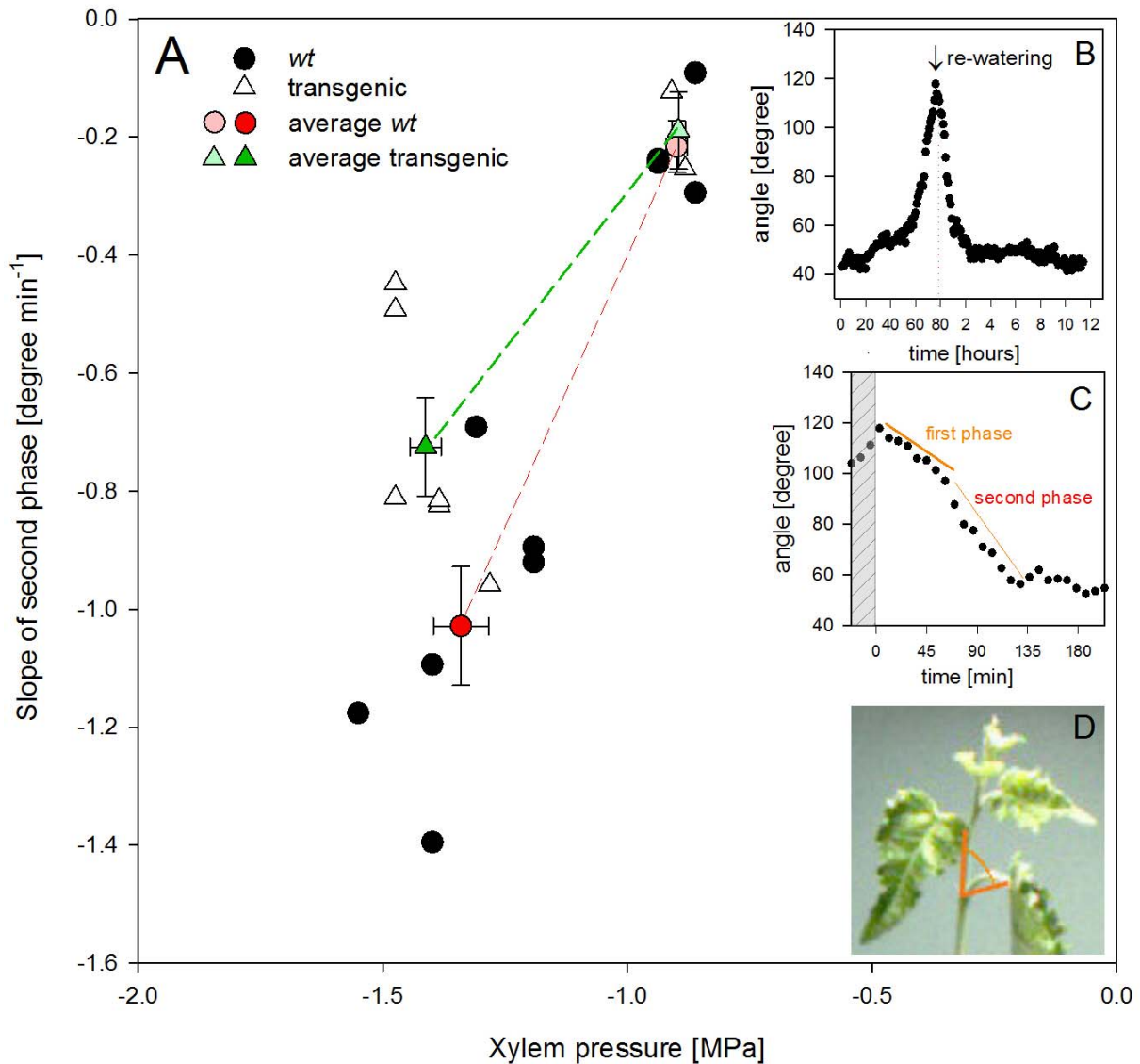


Figure 6

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.

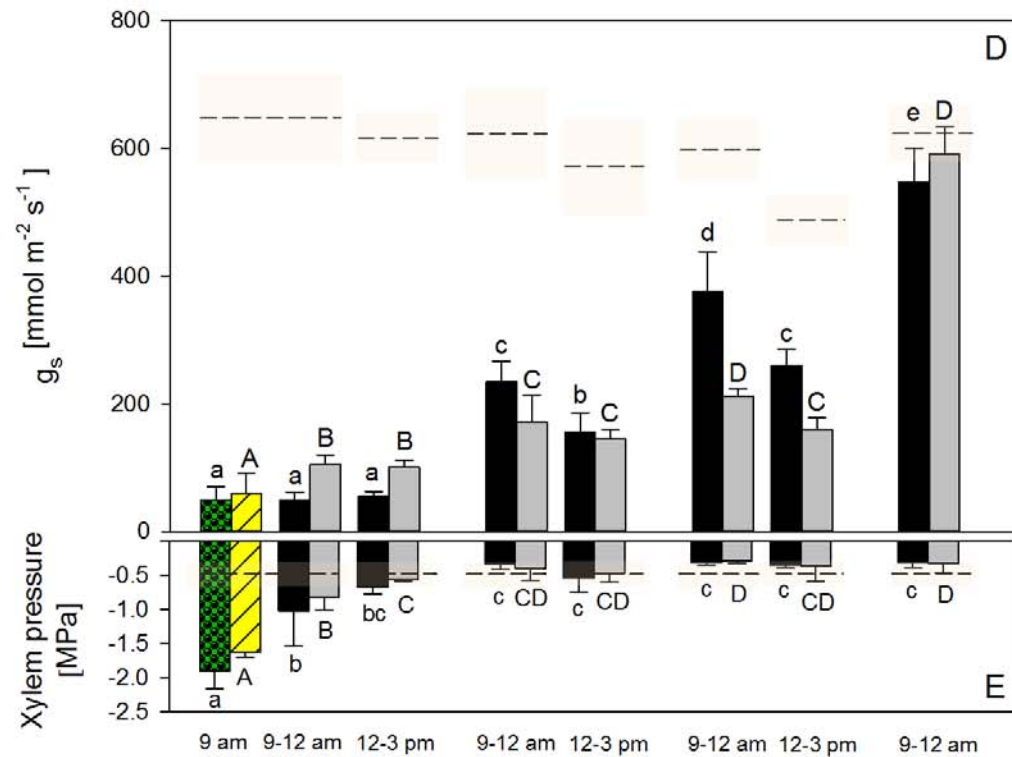
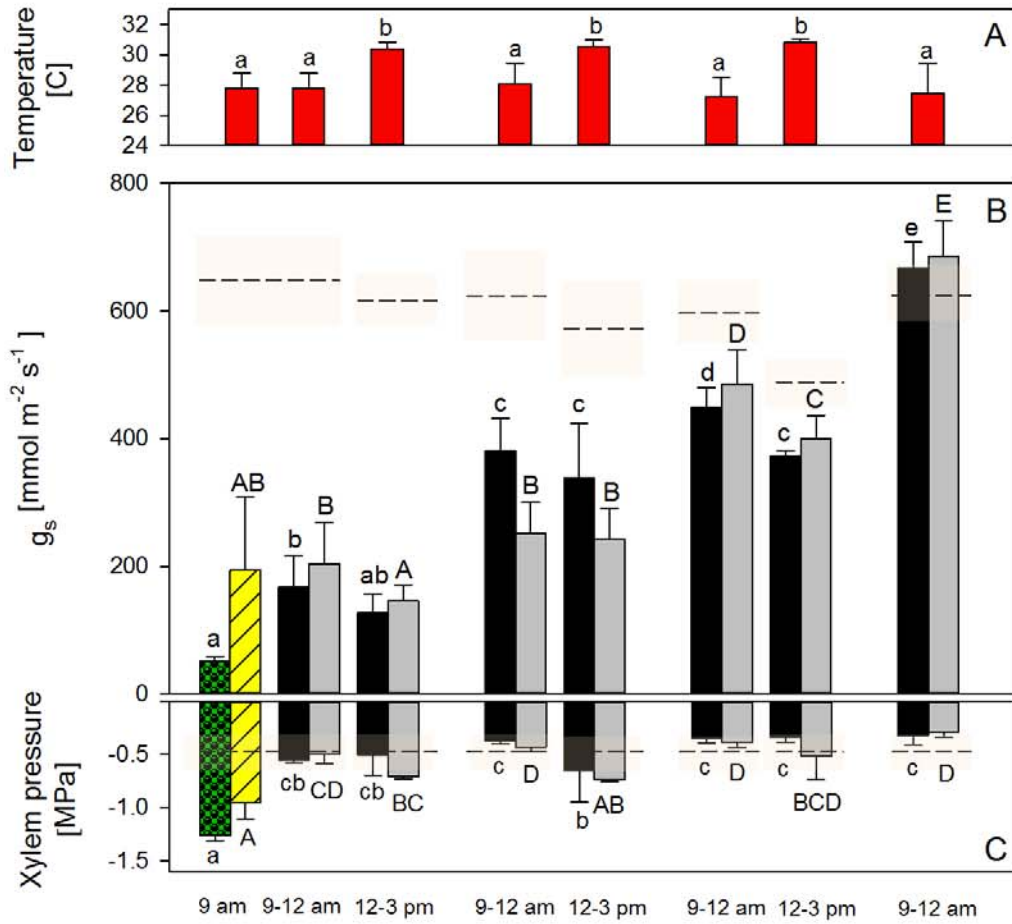


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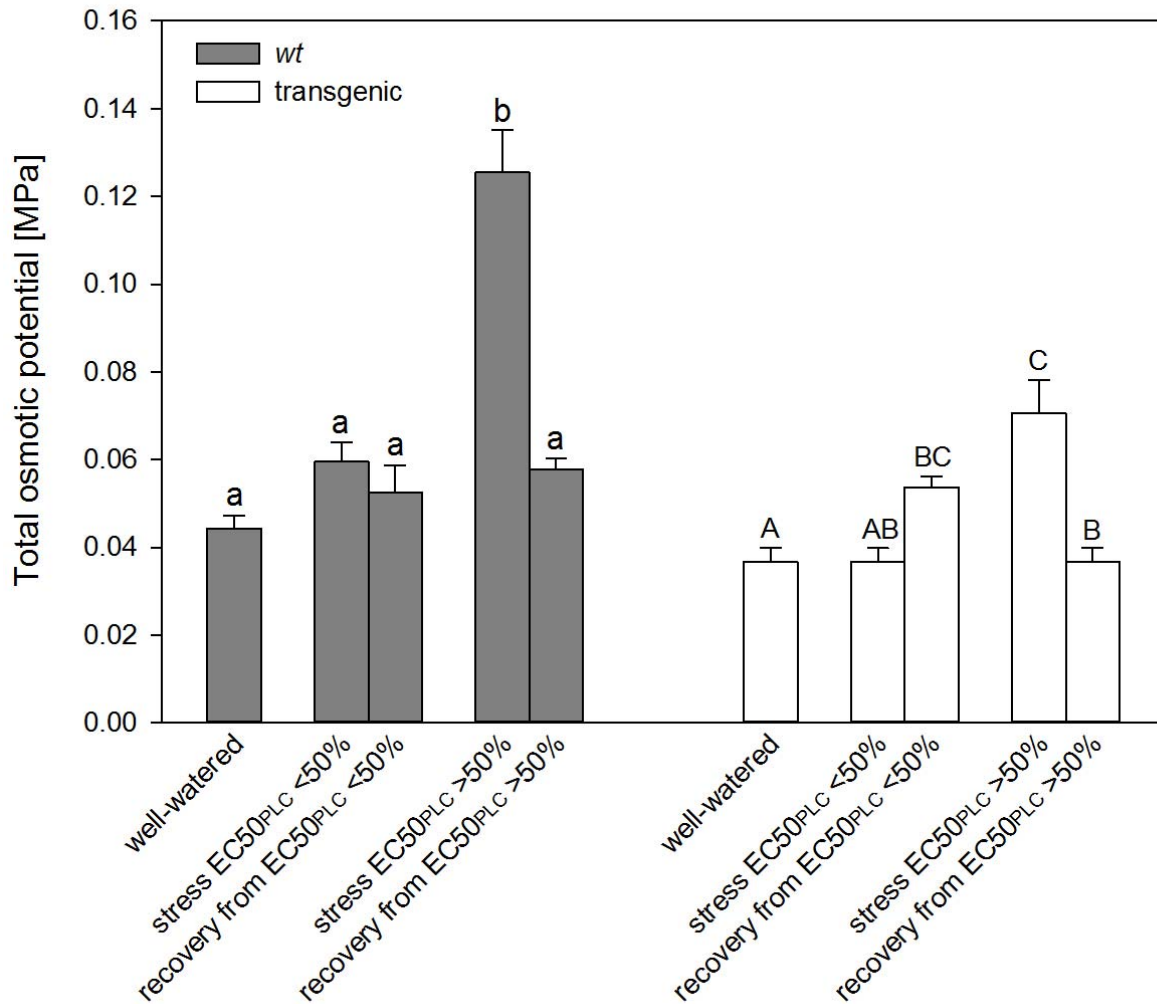


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Total osmotic potential collected from stem xylem sap of plants recovering from moderate ($EC_{50}PLC < 50\%$) and severe ($EC_{50}PLC > 50\%$) water stress. A one way Anova test suggests significant differences between treatments in both *wt* and 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.