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1 **VvPIP2;4N aquaporin involvement in controlling leaf hydraulic capacitance and resistance in**
2 **grapevine**

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27 **Abstract**

28 Hydraulic capacitance in a plant tissue (C), buffers the xylem tension storing and releasing water
29 and has been highlighted in recent years as an important factor that affects water relations such as
30 drought tolerance and embolism formation. Aquaporins are well known to control leaf hydraulic
31 resistance (Rh) but their role in the control of C is unknown. Here, we assess Rh and C on detached
32 grapevines leaves (cv. Brachetto) wild type (WT) and over-expressing the aquaporin gene
33 *VvPIP2;4N* (OE). For this purpose, we developed a new method inspired from the pressure-volume
34 curve technique and the rehydration kinetic method, which allowed us to monitor the dynamics of
35 dehydration and rehydration in the same leaf. The recovery after dehydration was measured in the
36 dark, in light non-transpirative conditions, light-transpirative conditions and transpirative condition
37 adding abscisic acid.

38 Pressurizing to dehydrate leaves in the OE line, the recorded Rh and C were respectively lower and
39 higher than those in the WT. The same results were obtained in the dark recovery by rehydration
40 treatment. In the presence of light, either when leaves transpired or not (by depressing vapour
41 pressure deficit), the described effects disappeared. The change in Rh and C did not affect the
42 kinetics of desiccation of detached leaves in the dark in air, in OE plants compared to WT ones.
43 Our study highlighted that both Rh and C were influenced by the constitutive over-expression of
44 *VvPIP2;4N*. The effect of aquaporins on C is reported here for the first time and may involve a
45 modulation of cell reflexion coefficient.

46
47 **Key words:** *Vitis vinifera* (L.), transgenic plant, leaf water potential, pressure-volume curve,
48 isohydric, anisohydric, osmoregulation.

49
50 **Abbreviations:**

51 AQPs, aquaporins; OE, over-expressing; WT, wild type; C, hydraulic capacitance; Rh, hydraulic
52 resistance; RWC, relative water content; Ψ , leaf water potential; **+0.5**, dehydration treatment with
53 pressure applied of 0,5 MPa; **+1**, dehydration treatment with pressure applied of 1 MPa; **dark**,
54 rehydration treatment in dark condition; **light VPD \approx 0**, rehydration treatment in light condition
55 without transpiration; **light transp**, rehydration treatment in light and transpirative condition; **light**
56 **transp ABA**, rehydration treatment in light transpirative condition supplying ABA in the
57 rehydrating water.

58

59

60 **Introduction**

61 Water is the most limiting resource for plant life and yield (Lange et al. 1982). Although the
62 majority of a plant's fresh weight consists of water, the amount of water retained by the plant in the
63 biomass is less than one percent of the total water transpired via stomata. Consequently, a huge
64 quantity of water is required to enable photosynthesis and plant growth. Therefore, water uptake
65 from the soil, its transport, storage and usage are mediated through a system that has evolved to
66 fully exploit the chemical and physical properties of water.

67 The cohesion-tension theory, formulated by Dixon (1914), explains the transport of water in the
68 soil-plant-atmosphere continuum. In this system, water moves from high to low water potential (Ψ),
69 and thus, towards transpiring leaves. Transpiration itself drives the rise of the xylem sap and
70 submits water to a considerable tension. Such tension is balanced by the hydrogen bonds among
71 water molecules, which prevent the breaking of the water column. However, under different
72 conditions (e.g., water shortage, freezing, high evaporative demand), this tension can increase and
73 cavitation can occur by air seeding mechanisms (Angeles et al. 2004).

74 In Dixon's theory, water transport in plants behaves like an electrical circuit, and follows Ohm's
75 law as described by van den Honert (1948), hence, the flow is due to the water potential gradient
76 and is hindered by the hydraulic resistance (R_h). Moreover, the pathway can be split into the water
77 transport of individual organs (root, stem, leaf), each with its own R_h that affects the water flux
78 (Tyree and Ewers 1991; Sperry et al. 1998). However, in this equation, the hydraulic capacitance
79 (C) has to be considered as an important variable that affects the output. Analogously to an
80 electrical circuit, C has the function of a capacitor (or condenser) used to store charge temporarily,
81 thus buffering a power surge. Therefore, in plants, C represents the ability to store water and to
82 buffer the system reducing the degree of tension in the xylem in transient water status conditions.
83 Capacitance corresponds to the ratio between the change in water content and the change in water
84 potential ($C = \Delta RWC / \Delta \Psi$; Tyree and Ewers 1991; Sperry et al. 2008); its effect is to make the
85 amount of water entering a region different from the amount of water leaving it, whenever $\Delta \Psi$
86 changes.

87 Leaves are the final component of the water transport system and via their stomata, they balance
88 carbon nutrition and water loss by transpiration, thereby playing a key role in the regulation of the
89 water status and the strategy of responses to drought stress. To prevent deleterious dehydration,
90 stomatal conductance is controlled by a complex regulation of guard cells, involving chemical and
91 hydraulic signals (Comstock 2002). The relevance of leaf C was recently investigated in relation to
92 various physiological traits, such as leaf thickness (Sack et al. 2003), leaf water content per unit dry
93 weight, leaf mass per unit area and lignin content (Blackman and Brodribb 2011). In addition, the

94 latter study described the ‘dynamic C’ (computed as the volume of flowing water measured by a
95 flowmeter) to be highly coordinated with leaf hydraulic conductance (Blackman and Brodribb
96 2011).

97 Aquaporins (AQPs) exercise a strategic function in the leaf water pathway by controlling
98 symplastic water movements (Kaldenhoff et al. 2008), and being the main link between the
99 symplastic and apoplastic pathways, e.g., bundle sheath cells. These water channels, without
100 changing the flux direction, can enormously increase the water movement across membranes, and
101 therefore, decrease the Rh. Aquaporins can be modulated at several levels, via transcription,
102 translation, trafficking and gating (opening and closing of the pore) and by environmental and
103 developmental factors (Chaumont and Tyerman 2014), such as: irradiation (Prado et al. 2013;
104 Lopez et al. 2013), transpiration (Sakurai-Ishikawa et al. 2011; Laur and Hacke 2013), circadian
105 rhythms (Hachez et al. 2008), abscisic acid (ABA) feeding (Shatil-Cohen et al. 2011; Pantin et al.
106 2013), auxin feeding (Péret et al. 2012) and shoot wounding (Sakurai-Ishikawa et al. 2011;
107 Vandeleur et al. 2014).

108 Several experiments using transgenic plants overexpressing or silencing AQP genes have been
109 performed (reviewed by Martínez-Ballesta and Carvajal 2014) and have demonstrated that the
110 transcriptional modulation of AQPs generally modifies the Rh, however, to date, no results exist
111 concerning the effects of AQPs on C.

112 In this study, grapevine plants over-expressing *VvPIP2;4N* (an aquaporin previously described by
113 Perrone et al. 2012, extremely efficient in facilitating cell-to-cell water pathways), were used to
114 assess the role of this AQP isoform on leaf Rh and C during leaf dehydration and recovery. The
115 hydraulic parameters were evaluated by a new method derived from the pressure-volume curve
116 (Tyree and Hammel 1972) and the rehydration kinetic technique explained by Blackman and
117 Brodribb (2011).

118

119 **Materials and Methods**

120 *Plant material*

121 The experiments were performed on leaves of potted ‘Brachetto’ grapevines; 10 wild-type (WT)
122 and 10 transgenic plants from line 16, which overexpressed *VvPIP2;4N* (OE), previously described
123 by Perrone et al. (2012). The 4-year-old plants (two buds pruned with bud-break in March, non-
124 grafted) were grown in a greenhouse on a mixture of peat–loam, under natural light and CO₂
125 concentration conditions. Plants were irrigated regularly according to their needs. In this
126 experiment, fully expanded, mature leaves were used.

127 *Assessment of Rh and C in the dehydration and rehydration processes*

128 To assess leaf Rh and C during dehydration, a method similar to the pressure-volume curve
129 technique was used (PV curve, Tyree and Hammel 1972), whereas for the rehydration phase, a
130 modified rehydration kinetic method (see C_{dyn} measurements, Blackman and Brodribb 2011) was
131 applied.

132 The new method proposed required the use of a high-precision balance (Mettler Toledo AT261
133 deltarange; Greifensee, CH) and a modified Scholander pressure bomb, inverted over the balance,
134 and controlled by an external manometer (Bourdon, FR; class 0.1). The cut surface of the petiole,
135 which passed through the sealing system of the pressure chamber, was immersed in a cylinder (50
136 mL), filled with deionised water, placed on the balance plate (Fig. 1). The balance plate was
137 isolated from the laboratory atmosphere and the relative humidity inside the balance chamber was
138 kept close to 100% using wet paper. By applying and releasing the pressure in the chamber, the
139 flow out/in of the leaf was measured by an increase or decrease in weight measured by the balance,
140 as explained below.

141

142 Dehydration phase (Fig. 1a):

143 Leaves were collected at 18.00. The petiole extremity, cut under water, was submerged in deionised
144 water in non-transpirative conditions (dark, sealed bag) overnight, to allow full leaf hydration.
145 During the following day (at 9.00; 12.00; 15.00), the leaves were removed from water and were
146 immediately placed in the pressure chamber. After measurement of the native water potential
147 (Ψ_{leaf}), the pressure chamber was upturned on the balance, placing the petiole in the 50-mL
148 cylinder. Starting from this steady state, the pressure was increased to a value of +0.5 (noted **+0.5**
149 thereafter) and +1.0 MPa (**+1**) and was kept constant; the pressure rose by 0.05 MPa per second
150 regulated by a needle valve. The mean native Ψ_{leaf} , after one night hydration in water, was -0.01
151 MPa for both WT and OE leaves.

152 This kind of measurement was also possible with water-stressed leaves, taking care to maintain the
153 pressure inside the chamber slightly lower than that balancing the leaf water potential. This was
154 necessary to avoid water uptake by the leaf and thus, to maintain a stable weight on the balance to
155 begin measurements.

156

157 Rehydration phase (Fig. 1b):

158 The pressure was applied to the dehydrated leaves until $\Psi_{\text{leaf}} = -1$ MPa (dehydration, **+1**). After
159 reaching this level of dehydration, the pressure was released and the chamber was removed. The
160 petioles remained suspended by the lid of the pressure chamber and immersed in the 50-mL

161 cylinder. The water uptake by the leaves began immediately after depressurisation and was
162 monitored for 1 h in different conditions:
163 1) dark and non-transpirative condition (**Dark**, leaf in a dark glass bell);
164 2) light and low transpirative condition (**Light VPD \approx 0**, a 2L glass baker was placed over the leaf
165 with artificial light set at 500 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$, wet paper was previously used to reduce the
166 VPD to 0 with formation of condensed water on the glass surface);
167 3) light and transpirative condition (**Light transp**, artificial light set at 500 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$, leaf
168 in laboratory atmosphere);
169 4) light, transpirative condition and ABA (**Light transp ABA**, conditions as in 3), and ABA
170 solution (100 μmol final concentration) supplied in the cylinder.

171 In the transpiring treatments, the air temperature was between 19°C and 24°C and the relative
172 humidity was between 45 and 60% (max VPD 10 Pa/KPa).

173 The Rh and capacitance C were obtained from the equation describing the pressure-volume curves
174 and was computed using the formula:

175
$$f(x) = P * C * 1^{(-x/(Rh*C))} \quad (\text{Tyree and Hammel 1972})$$

176 where: x is the cumulative water in/out of the leaf and P is the pressure applied. Using this equation
177 Rh correspond to the slope of the first part of the curve and C to the *plateau* phase, as highlighted in
178 Fig. 2.

179 To avoid errors in the calculation due to the transpiration, in the rehydration phase, C was computed
180 subtracting mathematically the transpiration rate from the water uptake weight measurements above
181 600 seconds. Until this time, transpiration marginally affected the water uptake (see Fig. 4). Data
182 were normalised to the dry weight (70°C, 12 h) and the leaf area (measured by the area-meter
183 Li3000, Lincoln NE, USA) of the single leaves. SigmaPlot 12.3 software (Systat Software, San Jose
184 CA, USA) was used for data elaboration and statistical analysis by *t*-test and one-way ANOVA
185 (after passed Shapiro-Wilk test). To perform ANOVA analysis, data were normalised when the
186 homogeneity of variance test (Bartlett's test) failed.

187

188 *Aquaporin expression profile*

189 Leaves for the aquaporin expression analysis were collected in light and dark conditions following
190 the same time-course and sampling protocol as for leaves used in the physiological tests (collected
191 at 18.00 and left rehydrated overnight in a dark, sealed bag). Leaves in the dark treatment were
192 harvested in liquid nitrogen at 9.00, whereas leaves in the light treatment were submitted to
193 artificial irradiation for 1 h before harvesting. The real-time RT-PCR (qRT-PCR) quantification of
194 transgenic *VvPIP2;4N*, endogenous *PIP2* genes and *PIP1*-type aquaporins were carried out as

195 previously reported (Perrone et al., 2012) on two biological replicates (three technical replicates
196 each).

197

198 *Leaf dehydration dynamics in dark conditions*

199 For each line, 22 leaves were sampled at 18.00; petioles were cut under water to avoid embolism
200 formation. Leaves were left free to rehydrate through the petiole in deionised water overnight, as
201 above. The following day at 8.00, water was removed and leaves were left dehydrated on the bench
202 in the dark in the laboratory atmosphere. The fresh weight of fully hydrated leaves was measured
203 with a balance (Denver Instruments Company TR603D; Arvada CO, USA), and then for each line,
204 the weight and Ψ_{leaf} were measured every hour during dehydration. The dry weight was recorded
205 after drying the leaves at 70°C for 12 h as above, and was used to calculate the relative water
206 content (RWC). The C was newly computed according to Koide et al. (2000), as:

$$C = \frac{\text{RWC}/\Psi_{\text{leaf}}}{\text{dry weight}}$$

207

208 **Results**

209 *Dehydration phase*

210 During dehydration, water was forced to exit through the petioles, and a higher flow out through the
211 petiole of the OE leaves was observed in comparison to the WT, as shown in Fig. 2, when leaves
212 were pressurised to +0.5 MPa (+0.5). These differences were also observed if the data were
213 normalised by the leaf dry weight or leaf area (Fig. 2).

214 By collecting the data during three different daily time-points (Fig. S1); morning, noon and
215 afternoon, we observed that for both lines, the amount of water that exited from the petioles
216 followed an increasing trend and reached a maximum at noon and a minimum in the morning.
217 The mean Rh and C, obtained by pressurising leaves (dehydration phase), are shown in Fig. 3 (and
218 Fig. S3). When leaves were pressurised to +0.5 MPa (+0.5), Rh was very low in both lines with no
219 significant differences, whereas at +1 MPa (+1), the Rh increased drastically and a significant
220 difference was observed in the OE line ($P < 0.01$), where the Rh was lower than in WT (Fig. 3a).
221 Conversely, C was significantly higher (+38%, $P < 0.05$) in the OE line in the first treatment (+0.5),
222 but this difference disappeared when the pressure was increased to +1 MPa (+1) (Fig. 3b).

223

224 *Rehydration phase*

225 Subsequent to dehydration to $\Psi_{\text{leaf}} = -1.0$ MPa (+1 treatment), leaves were left to rehydrate for 1 h
226 (rehydration phase) by subjecting them to different stimuli. The total time course of the amount of
227 water flowing into the petiole after the pressure release is shown in Fig. 4. Moreover, the first 600

228 seconds of the experiment, which was used to compute Rh and C, are highlighted in Fig. 4 frame **b**.
229 These figures illustrate that for all lines and conditions, the recovery from stress occurred via a slow
230 rise in the volume of water absorbed, and that transpiration began at about 600 s following
231 depressurisation, as suggested by the divergence among treatments with (**light transp**, **light transp**
232 **ABA**) or without transpiration (**dark**, **light VPD≈0**). Finally, although the standard errors
233 overlapped for all treatments, the leaves of the WT line in dark conditions appeared to behave
234 differently from those in the other treatments, which uptook more water.

235 The recovery behaviour following dehydration can be analysed by Rh, C (calculated from the
236 dynamics shown in Fig. 5) and the Ψ_{leaf} reached after 1 h of rehydration (Table 1). Major
237 differences in Rh between the two lines were observed in dark conditions (Fig. 5a), where in WT
238 leaves, the Rh was significantly higher than in OE leaves ($P < 0.01$). This result agrees with the
239 difference observed in the +1 treatment (where dark conditions were ensured by the pressure
240 chamber) between WT and OE, although at a higher order of magnitude. The switch from **dark** to
241 **light VPD≈0** conditions decreased the Rh in WT to levels similar to those in the OE line, whereas
242 Rh was not affected by the transition between dark and light in OE leaves. Transpiration (**light**
243 **transp** treatment) did not have any effect on the Rh in WT lines, whereas a slight increase was
244 observed in the transgenic line. Finally, after the addition of ABA to the solution absorbed through
245 the petiole (**light transp ABA**), the Rh in WT leaves increased, but not significantly, compared to
246 other rehydration conditions in the light and from the transgenic line. However, in general, we
247 observed a reduction in Rh in WT following the transfer from the dark to the light conditions
248 adopted in the rehydration experiments, whereas the Rh in the OE line tended to increase with
249 increased transpiration.

250 The C computed from the same dataset did not differ between WT and transgenic leaves; only the
251 dark condition strongly affected this parameter, causing it to be significantly lower ($P < 0.001$) in
252 the WT than in all other treatments.

253 The Ψ_{leaf} recorded in the pressurisation experiment and its recovery are reported in Table 1. During
254 dehydration, there were no differences in the native Ψ_{leaf} and consequently in the final Ψ_{leaf} reached.
255 However, in contrast, during rehydration, the leaves of the two lines revealed a different ability to
256 recover the Ψ_{leaf} within 1 h after de-pressurisation. In particular, both lines in dark conditions
257 showed a higher recovery rate, reaching a Ψ_{leaf} close to 0. On the contrary, Ψ_{leaf} decreased when
258 leaves were subjected to artificial light and transpiration, whereas ABA treatment facilitated the
259 recovery of Ψ_{leaf} . The statistical analysis showed differences in the Ψ_{leaf} between the two lines in the
260 **light VPD≈0** and **light transp** treatments.

261 Based on these observations, the expression levels of transgenic *VvPIP2;4N*, together with those of
262 other known *PIP2* genes and a PIP1-type aquaporin were quantified by qRT-PCR in dark and light-
263 transpiration conditions in both lines. The WT showed the same AQP expression profile in dark and
264 light-transpiration conditions, suggesting a light-independent expression of these *PIP* genes (Fig.
265 6). Furthermore, in the OE line, the expression profile of AQPs and transgenic *VvPIP2;4N* was
266 generally not affected by light; only *VvPIP2;2* was slightly more highly expressed in the dark.

267

268 *Leaf dehydration dynamics in dark conditions*

269 The dynamics of the dehydration of detached leaves in darkness was observed from the relationship
270 between Ψ_{leaf} and relative water content (RWC). In addition, C was calculated as $\Delta\text{RWC}/\Delta\Psi^*\text{dry}$
271 weight. The linear regression indicated a slightly higher RWC coupled to the decrease in Ψ_{leaf} in
272 OE compared to WT leaves (Fig. 7a). The hyperbole describing Ψ_{leaf} versus C was similar in both
273 lines, showing a reduction of C that was related to the decrease in Ψ_{leaf} (Fig. 7b). Overall, the mean
274 values of C for the two lines confirmed the higher C in OE lines (156 ± 26 for the WT, 261 ± 52 for
275 OE; $P < 0.05$).

276 Finally, to evaluate whether the observed differences were attributable to anatomical or
277 morphological traits, the pairwise relationships between leaf area, dry weight and fresh weight were
278 assessed, without identifying any significant differences between WT and OE samples (Fig. S2).

279

280 **Discussion**

281 In this study, transgenic grapevines that constitutively over-expressed *VvPIP2;4N* under the
282 *Cauliflower mosaic virus* 35S promoter (Perrone et al. 2012) were used to assess the role of this
283 AQP on leaf Rh and C during leaf dehydration and recovery. Many studies in several transgenic
284 plants had previously shown that overexpression of aquaporin genes decreased the Rh (Ding et al.,
285 2004; Lee et al. 2012; Perrone et al. 2012), whereas the silencing of AQPs resulted in an increase in
286 Rh (Siefritz et al. 2004, Sade et al. 2014). However, no information is available concerning the
287 relationship between AQP and C.

288

289 *The effect of aquaporins on hydraulic resistance (Rh)*

290 As expected, the Rh was lower in OE leaves than WT leaves when a high over-pressure was applied
291 to the leaves (+1) and when recovery was performed in **dark** conditions. These two results can be
292 ascribed to a direct effect of transgenic *PIP2;4N*, since an increase in *PIP2;4N* protein in the
293 membranes improves the membrane permeability to water.

294 Several studies have demonstrated that AQPs expression and activity are regulated in leaves by
295 circadian rhythms (Siefritz et al. 2002; Nardini et al. 2005; Hachez et al. 2008). In this study, the
296 dynamics of the cumulative water outflow from leaves showed an influence of circadian rhythms
297 both in OE and WT leaves (Fig. S1). These differences might have affected the computation of the
298 hydraulic traits (Fig. S3); however, to limit the impact of the biological clock, the experiments were
299 performed at distinct times during the day and averaged together in both genotypes. AQP
300 expression has been assessed just during the morning. However, the impact of circadian rhythms on
301 the extremely high expression of the transgene (meanly 7 times higher than endogenous aquaporins)
302 can be reasonably neglected. In addition, it is known that the 35S gene promoter, controlling
303 expression of our transgene, shows low or no sensitivity to the biological clock (Millar et al. 1992;
304 Xu and Johnson 2001).

305 The leaf Rh increase with increased dehydration as observed in Fig. 3, where Rh drastically
306 increased from the +0.5 to the +1 treatment, which was reported previously (Sack & Holbrook
307 2006; Scoffoni et al. 2014). However, this might represent a physical artefact. One hypothesis might
308 be that a pressure of 1 MPa leads to a massive flow of water in the leaf hydraulic system in a short
309 time interval. Probably, the anatomy of the leaf itself (e.g., connectivity between cells, bundle-
310 sheath permeation, petiole conductivity) hinders the runoff of a large amount of water in very short
311 period, leading to an overestimation of the Rh. This phenomenon might explain the different
312 magnitude of the Rh values in the dehydration +1 and recovery treatments.

313 In the recovery trial (rehydration, Fig. 6), the impact of various stimuli, such as i) light, ii) ABA and
314 iii) transpiration on the aquaporin activity was studied.

315 i) The light effect cancelled the differences in Rh observed in the **dark** between WT and OE.
316 Indeed, in WT, the hydraulic resistance decreased from dark to light conditions, whereas this
317 parameter was not affected in OE leaves. This change in the Rh in WT leaves agrees with the
318 increase in leaf conductivity under irradiation previously reported by several authors (Nardini et al.
319 2010, Sellin et al. 2010; Guyot et al. 2012; Lopez et al. 2013; Prado et al. 2013). Cochard et al.
320 (2007) indicated two potential light-modulated mechanisms of water movement in leaves: activated
321 AQPs in light conditions allows water to move freely in the symplast and apoplast, whereas at low
322 irradiance, deactivated AQPs force the water to move apoplastically, limited by the bundle sheath.
323 Voicu et al. (2009) highlighted that the light-dependent change in leaf hydraulic conductance in bur
324 oak (*Quercus macro-carpa*) was not linked to any AQP transcriptional changes. Similarly, in this
325 study, we observed only slight differences in the expression profile between WT and OE following
326 changes in the light conditions (Fig. 6). The qRT-PCR data showed clearly that the major difference
327 between WT and OE lines derived exclusively from the high and constitutive expression of

328 transgenic *VvPIP2;4N* in all conditions (Figure 6). Thus, for the OE line, the low levels of Rh in
329 dark conditions were probably linked to the constitutive over-expression of *VvPIP2;4N*. Some type
330 of contrasting regulation can be hypothesised between the light-mediated activation of leaf AQPs
331 (as in WT) and *VvPIP2;4N*. In WT plants, light activates AQPs depressing the Rh recorded upon
332 dark condition, whereas in OE plants the effect of *VvPIP2;4N* (a root-specific AQP isoform,
333 presumably insensitive to light modulation) is masked from the light-activation of the other leaf
334 aquaporins.

335 ii) ABA modulates AQP activity, having opposite effects on root and leaf AQPs:
336 downregulating the bundle-sheath AQPs and thus limiting hydraulic conductivity in leaf (Pantin et
337 al. 2013; Shatil-Cohen et al. 2011), and upregulating the AQP isoforms and increasing the hydraulic
338 conductivity in the root (Jang et al. 2004; Hose et al. 2000; Thompson et al. 2007; Parent et al.
339 2009). In this study, no significant differences in Rh between OE and WT were observed in the
340 presence of ABA. However, comparing the **light transp** and **light transp ABA** treatments, Rh
341 values were twice as high in WT, even if this difference was not statistically significant, whereas no
342 changes were observed in OE leaves. We can speculate that ABA caused a closed conformation of
343 PIP2;4N or inhibited the expression of all AQP isoforms in WT leaves. This downregulation might
344 also occur in OE leaves, but additionally and in contrast, ABA might promote the expression or
345 open conformation of PIP2;4N. The PIP2;4N protein is a root-specific AQP, and therefore, is
346 putatively upregulated by ABA; its presence might have led to a lack of increase in Rh. The first
347 ABA signalling transduction pathway that mediates water transport in roots has been recently
348 demonstrated in maize (Fan et al. 2015). Notably, the post-translational regulation of ZmPIP
349 through ABA signalling appears to be particularly important to regulate root hydraulic conductivity.
350 This might also be the case for transgenic *VvPIP2;4N* aquaporin in leaf: since it is under the control
351 of a constitutive promoter (35s), ABA might promote its activity in a phosphorylation-dependent
352 manner. Moreover, Chitarra et al. (2014) demonstrated that ABA promoted *VvPIP2;4N* expression
353 in vessel-associated cells (VACs), but not in whole petiole tissue. Similarly to the VACs, the leaf
354 bundle sheath cells regulate exchange between the xylem and other parenchyma cells. The ABA-
355 induced upregulation of *VvPIP2;4* at this level might easily explain the lack of rise of Rh level in
356 the transgenic leaves.

357 A secondary effect of ABA was observed in the recovery of the final Ψ_{leaf} after 1 h of rehydration
358 (Table 1). In contrast to the **Light trans** treatment (final $\Psi_{\text{leaf}} = -0.23$ MPa for WT and -0.35 MPa
359 for OE), ABA promoted a more rapid recovery of Ψ_{leaf} in OE leaves than WT leaves (-0.19 MPa for
360 WT and -0.17 MPa for OE). A positive effect of ABA on Ψ_{leaf} recuperation has been already
361 described by Lovisollo et al. (2008) and Chitarra et al. (2014) in grapevine. In the OE leaves, the

362 better recovery of Ψ_{leaf} appeared to be coupled to a low hydraulic resistance, in agreement with data
363 reported by Martre et al. (2002).

364 iii) In this study, we observed no effect of transpiration on changes in leaf Rh, contrary to that
365 reported for AQPs in root (Sakurai-Ishikawa et al. 2011; Laur and Hacke 2013). This might be due
366 to the low vapour-pressure deficit in the laboratory atmosphere or to the real absence of an effect of
367 this parameter in leaf.

368

369 *The effect of aquaporins on hydraulic capacitance (C)*

370 The C plays an important role in drought tolerance, as does Rh, by affecting the amount of water
371 destined to buffer the change in the transpiration stream elicited by the atmospheric conditions and
372 especially the ability to extend survival after stomatal closure (Bartlett et al. 2012; Gleason et al.
373 2014). Since sapwood has been recognised as the major source of stored water, several studies have
374 addressed the importance of C in this tissue in depth. Generally, C varies in sapwood between 40
375 and 900 kg m⁻³ MPa⁻¹ (Scholz et al. 2007; Čermák et al. 2007; McCulloch et al. 2014) and is
376 inversely related to the wood density. The contribution to the total daily transpiration reported in
377 literature varies between 5% and 45% (Goldstein et al. 1997; Phillips et al. 2003; Verbeeck et al.
378 2007). However, the importance of the leaf C was highlighted by Gleason et al. (2014), who
379 suggested that the majority of water lost during dehydration derives from leaves. By comparing the
380 C in grapevine leaves in this study (up to 250 mg H₂O gDW⁻¹ MPa⁻¹), classifies them within the
381 upper half of the ranking proposed by Blackman and Brodribb (2011), among species with a low C,
382 although the measured units are not the same (the conversion was performed by considering that 1
383 m² of leaf area corresponds to 31 g DW, from Fig. S2).

384 A novel aspect highlighted by this study is the effect of AQP on C, which, as far as we know, has
385 never been reported so far. The ability of a tissue to be a capacitor, and to buffer the xylem tension
386 and prevent embolism was debated as an important trait that might distinguish plants and their
387 susceptibility to water stress (Sperry et al. 2008; McCulloch et al. 2014).

388 In the pressurisation tests, differences in C were observed between WT and OE leaves only when
389 the pressure applied was low (+0.5 MPa, Fig. 3b), in contrast to what was observed for Rh (Fig. 3a).
390 The absence of effect when the applied pressure reached +1 MPa might be due to the high
391 dehydration imposed on the leaves. Indeed, C is a variable parameter that decreases together with
392 water status (Fig. 7b), potentially becoming comparable in the two lines when the leaves were
393 dehydrated to -1 MPa.

394 In the recovery trials (Fig. 5b), WT leaves showed the lowest C in dark conditions compared with
395 the other treatments; probably, the high Rh observed in WT hindered the water uptake and thus, the

396 recovery of C. In theory, a longer recovery time might lead to the complete recovery of C in WT
397 leaves, although the leaves were in dark conditions. However, in dark conditions, the final Ψ_{leaf}
398 values fully recovered in both lines; even though this occurred in WT without a complete recovery
399 in C. Thus, in this latter case, the amount of water inside the WT leaves was lower than that in OE
400 leaves, and probably, the amount of water outflow pressurizing once more the leaf could be lower
401 than that observed during the first pressurisation.

402 The aim of the last experiment was to check whether OE leaves dehydrate more rapidly in dark
403 conditions (where major differences between WT and OE leaves were observed). However, the
404 overexpression of *VvPIP2;4N* did not lead to a more rapid leaf dehydration, and the computed C
405 ($\Delta\text{RWC}/\Delta\Psi \cdot \text{dry weight}$) confirmed higher values in the OE line.

406

407 *Hypothesis and ecological significance*

408 Using transgenic plants and the novel method proposed in this study, a positive relationship between
409 C and AQPs in grapevine leaves was demonstrated. The mechanisms underlying this interaction are
410 not yet clear, however, an initial hypothetical mechanism might involve the reflection coefficient
411 (σ) of the plasma-membrane. This parameter is considered to be the ability of a channel (AQP in
412 our case) to be permeable to, or reflect a solute. It is determined by the arginine selectivity filter at
413 the end of the pore (Zeuthen et al. 2013) and by the solute size. In the membrane of transgenic
414 plants in this study, the higher concentration of AQPs can lead to a higher permeation of small
415 solutes (Gomes et al. 2009), resulting in a low reflection coefficient, and consequently allowing a
416 higher water flow (coupled to osmolyte flow) through the lipid bilayer. Although this consideration
417 depends on the contribution of *VvPIP2;4N* to the total water transport across the plasma membrane.
418 The theoretical framework for the implication of σ on C is provided as supplemental information.
419 Recently, Maurel et al. (2015) proposed a pivotal role for AQPs in buffering cell osmoregulation,
420 by reviewing and re-interpreting AQP function as osmo-sensor in guard cells during stomatal
421 movements or in growing pollen tubes. The mechanism was only speculated, but an AQP-mediated
422 increasing cell C, conferring to the cells higher buffer capacitance, could give light to this still un-
423 described phenomenon.

424 A second hypothesis, which does not exclude the first, is that AQPs might connect several cells, and
425 increase the volume of the reservoir. In grapevine, AQPs can increase the link between the symplast
426 and apoplast in the areoles, the enclosed areas between the interconnected veins, thereby improving
427 the leaf capacitance. In figure 8 a schematic representation of the leaf hydraulic pathway is
428 represented with its simplification (right side), showing the increase of the total capacitor due to the
429 sum of different capacitors.

430 Previously, studies have attributed a role to AQPs in iso/anisohydric behaviour, due to changes in
431 leaf conductance (Sade et al. 2009; Vandeleur et al. 2009; Chaumont and Tyremann 2014). In other
432 studies (Ogasa et al. 2013; McCulloh et al. 2014), plants are categorised according to their C, for
433 their investment in structural features to maintain the transpiration stream (anisohydry) or their
434 sensitivities to embolisms (Tombesi et al. 2014).
435 Perrone et al. (2012) considered the ‘Brachetto’ WT as an anisohydric cultivar, and the transgenic
436 lines could be interpreted as being even more anisohydric. In this study, AQPs conferred a greater
437 C, and hence, a greater degree of anisohydry, highlighting the possible link between C, Rh,
438 aquaporins and iso/anisohydric responses to water stress. Clearly, the implication of Rh and C on
439 whole leaf hydraulics deserves further attention.

440

441 **Author Contributions**

442 H.C., C.L. and M.V. conceived and planned the study. M.V. performed the experiment and
443 analysed the data. M.V. wrote the first draft of the manuscript. G.G. and I.P. produced plant
444 materials, carried out the molecular analysis and reviewed the manuscript. A.P. helped in the
445 physical dissertations. H.C. theorized the hypothesis presented and reviewed the manuscript. C.L.
446 reviewed the manuscript and obtained funds to support the project.

447

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454

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632

633 **Captions to figures and tables**

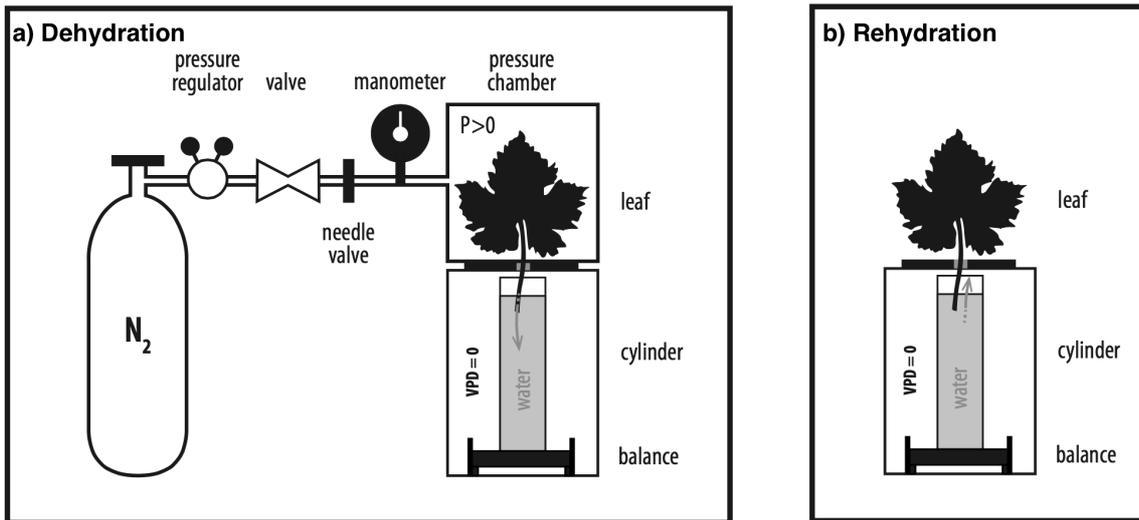
634 **Table 1:** leaf water potential (Ψ_{leaf} , MPa) \pm SE before and after the pressurisation and rehydration
 635 treatments. ANOVA followed by Tukey's post-hoc test was applied to assess significant differences
 636 among lines and treatments in the final Ψ_{leaf} obtained (* = $P < 0.05$). The experiments concerned
 637 two phases: in the first phase, the leaves were pressurised in the pressure chamber and water
 638 flowing out from the leaf through the petiole was measured; in the second phase, the pressure was
 639 released and water inflow into the leaf was measured by the weight-loss of water in the cylinder.

	Treatment	Line	native Ψ_{leaf}	Pressure applied	Final Ψ_{leaf}	ANOVA	n=
Dehydration phase	+0.5	WT	-0.01 \pm 0	+0.5	-0.5 \pm 0		6
		OE	-0.01 \pm 0	+0.5	-0.5 \pm 0		
	+1	WT	-0.01 \pm 0	+0.99	-1 \pm 0		16
		OE	-0.01 \pm 0	+0.99	-1 \pm 0		
Rehydration phase	Dark	WT	-1 \pm 0	none	-0.08 \pm 0.01	d	4
		OE	-1 \pm 0	none	-0.08 \pm 0.01	d	
	Light VPD\approx0	WT	-1 \pm 0	none	-0.20 \pm 0.02	b	4
		OE	-1 \pm 0	none	-0.14 \pm 0.01	c	
	Light trans	WT	-1 \pm 0	none	-0.23 \pm 0.01	b	4
		OE	-1 \pm 0	none	-0.35 \pm 0.08	a	
Light trans ABA	WT	-1 \pm 0	none	-0.19 \pm 0.09	b	4	
	OE	-1 \pm 0	none	-0.17 \pm 0.04	bc		

640

641

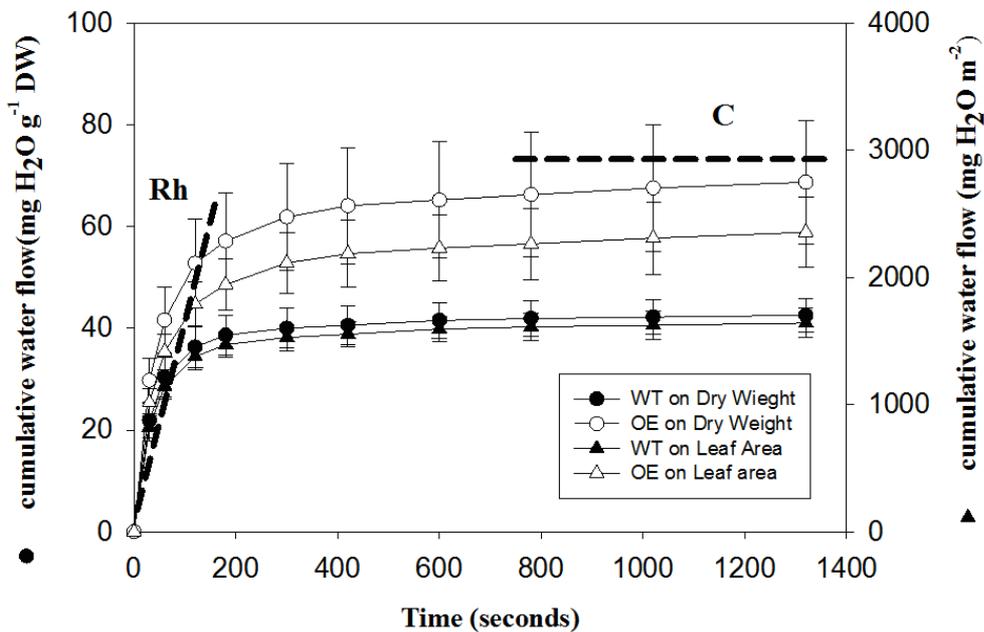
642 **Figure 1:** the experimental setup used in this study. Nitrogen gas, regulated through valves and
 643 monitored by a manometer, was used to compress the leaf in the pressure chamber. Water flowing
 644 out from the petiole increased the weight of the water-filled cylinder (left). Following rehydration
 645 (right), the chamber was removed, allowing the rehydration of the previously pressurised leaf. The
 646 rehydration was conducted under different stimuli (light, transpiration, ABA).
 647



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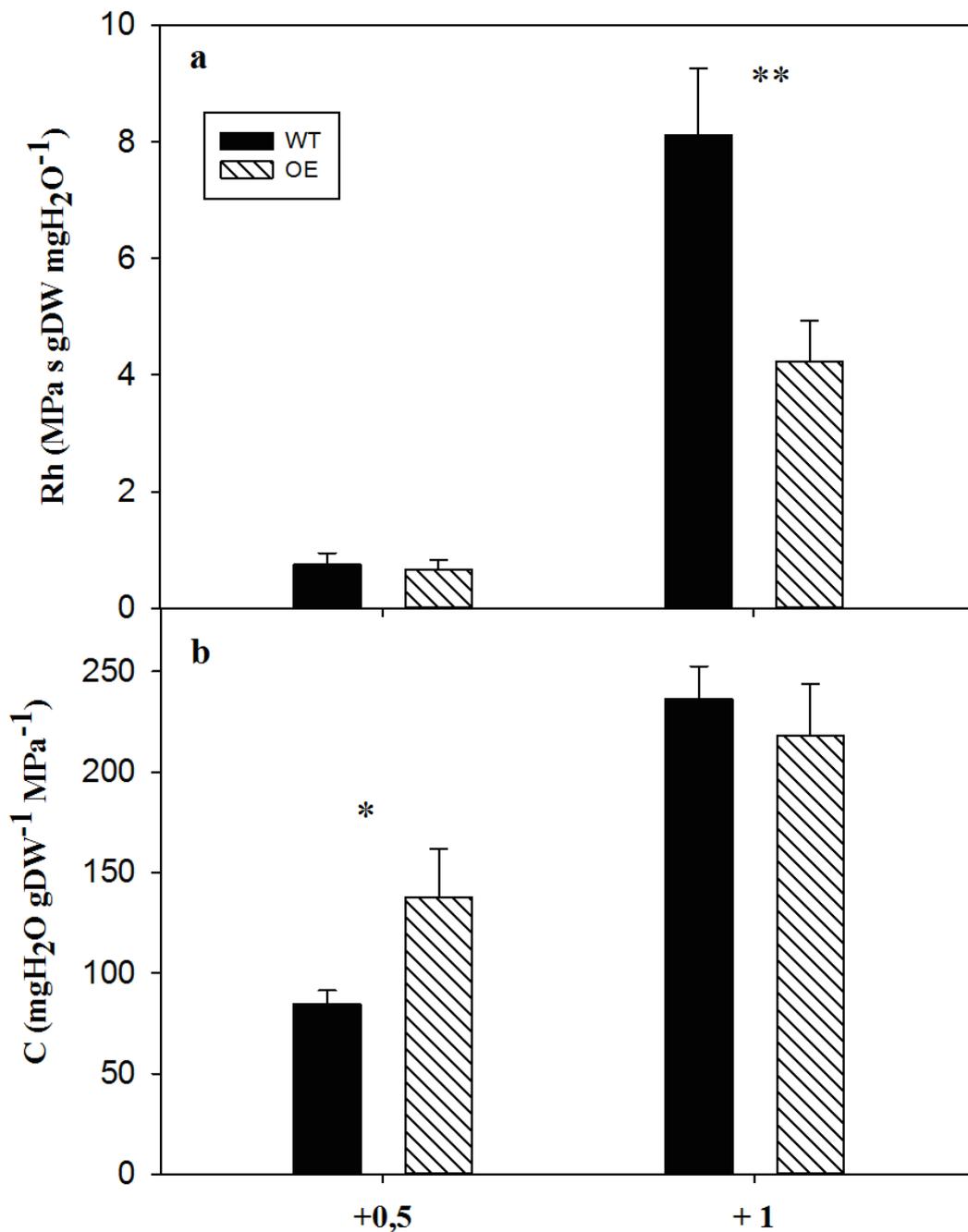
649

650 **Figure 2:** time-course of the cumulative water flow out of leaves in WT leaves (filled symbols) and OE
 651 leaves (empty symbols) in the +0.5 experiment. Data were normalised according to the dry weight (circles,
 652 left y-axis) or leaf area (triangles, right y-axis). Symbols represent the means \pm SE. (n = 6).



653

654 **Figure 3:** a) hydraulic resistance (Rh), and b) capacitance (C) in WT leaves (black columns) and
 655 OE leaves (grey columns) obtained by pressurising the leaves as described in dehydration phase fo
 656 the experimental design: rehydrated leaves pressurised to +0.5 MPa (+0.5) or +1.0 MPa (+1).
 657 Columns represent the means ($n = 6$ for +0.5 and $n = 16$ for +1.0) \pm SE Means were obtained by
 658 averaging the measurements performed at different times of day (8:00–10.00; 11:00–13.00; 14:00–
 659 16.00). Asterisks mark significant differences between means (* = $P < 0.05$ ** = $P < 0.01$).

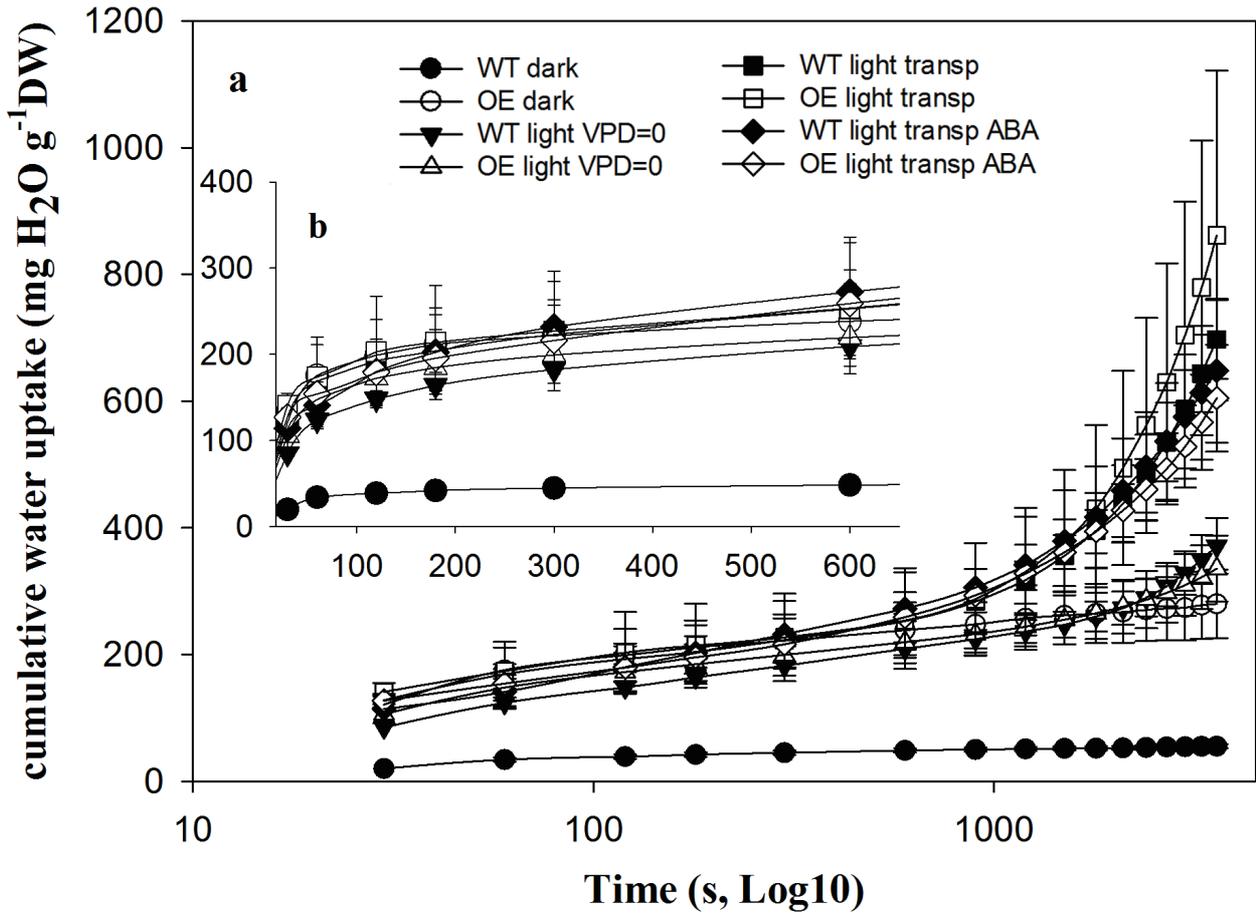


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661

663 **Figure 4:** frame a; time-course of the water flow into the petioles of WT leaves (filled symbols) and
 664 OE leaves (empty symbols) in the four recovery treatments: dark (circles), light low transpirative
 665 conditions ($VPD \approx 0$; triangles), light transpirative conditions (square) and light transpirative
 666 conditions in the presence of ABA (rhombus) ($n=4$). Frame b highlights the first 600 s of the time-
 667 course (the mean values of of Rh and C are displayed in Fig. 5).

668

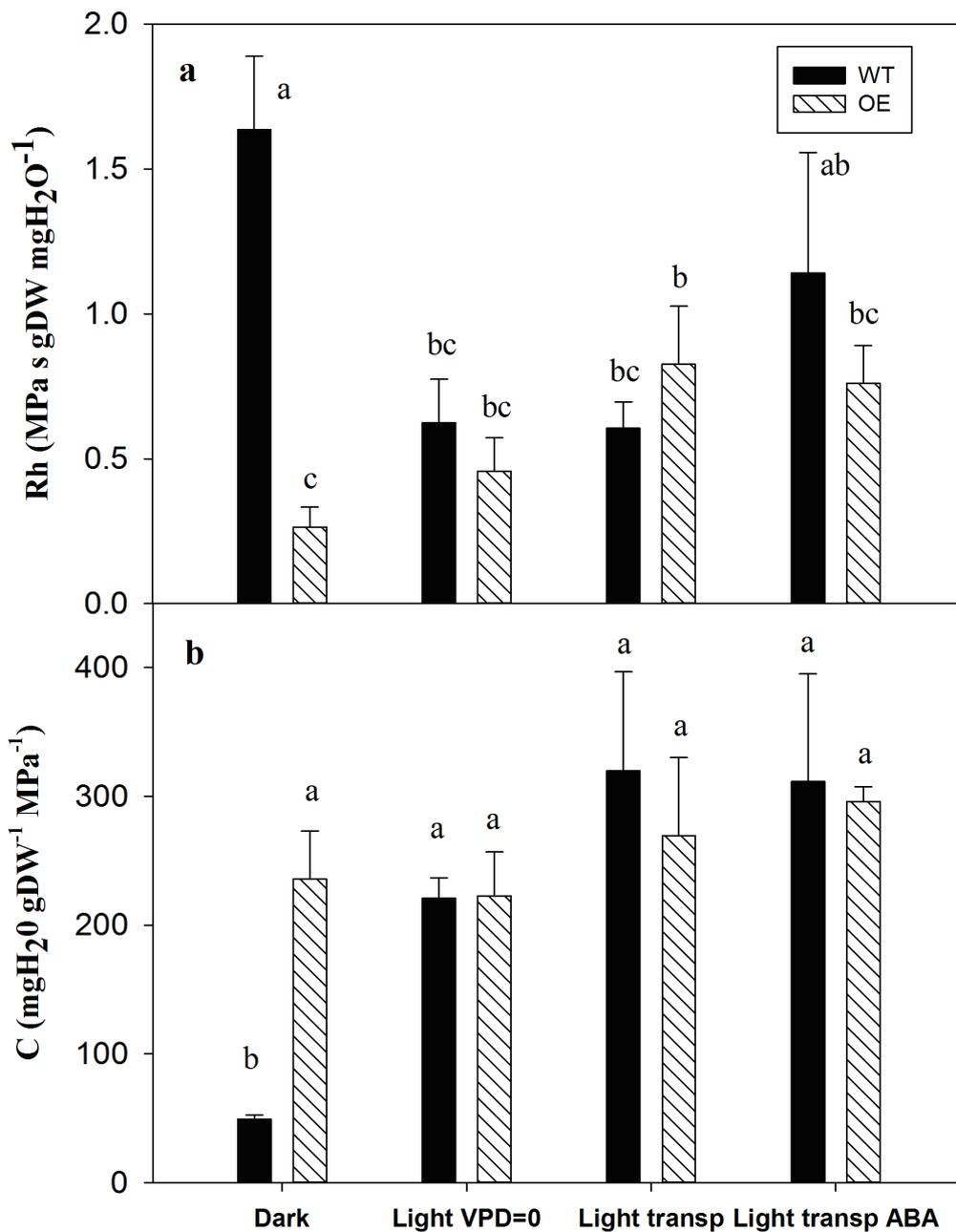


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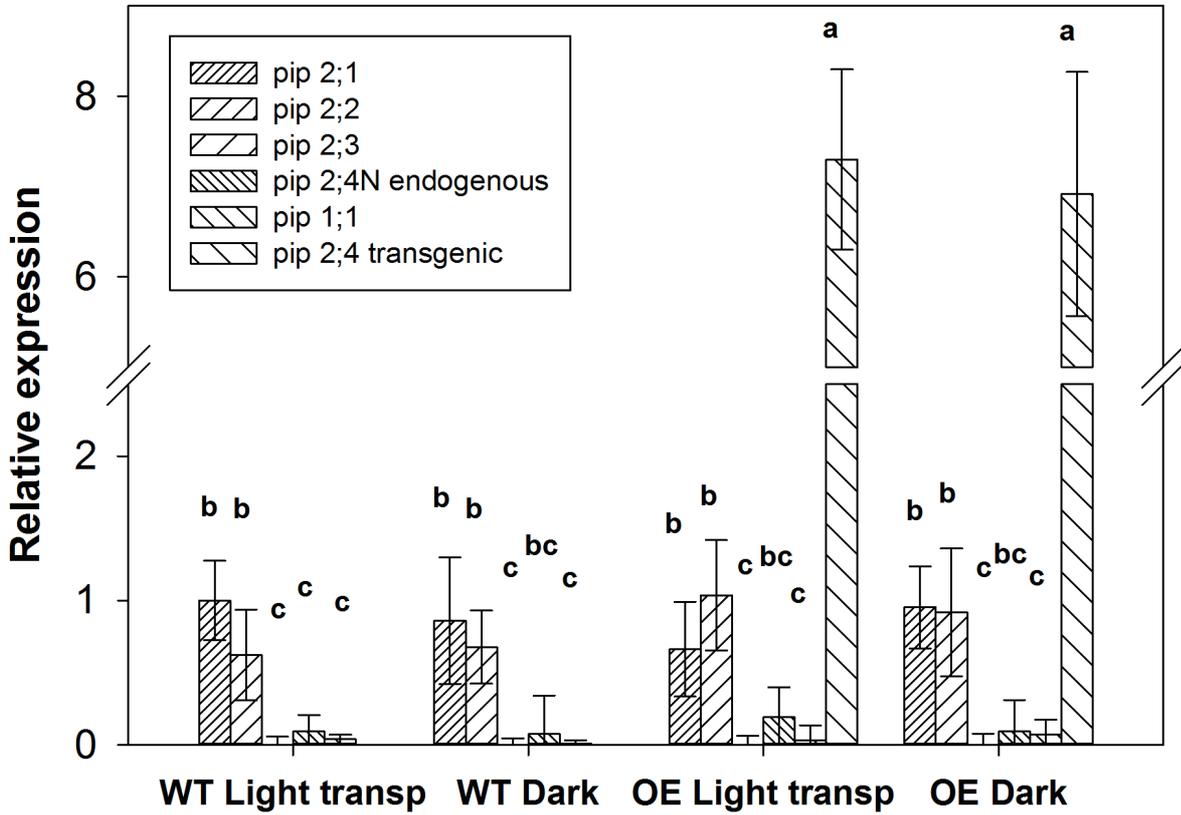
672 **Figure 5:** a) hydraulic resistance (Rh), and b) capacitance (C) in WT (black columns) and OE (grey
673 columns) obtained from the rehydration of leaves after dehydration to $\Psi_{\text{leaf}} = -1$ MPa (+1), as
674 described in the rehydration phase of the experimental design. Recovery treatments were performed
675 in dark, in light low transpirative, light transpirative or light transpirative conditions after adding
676 ABA (final concentration 100 μmol) to the cylinder where the cut petioles were submerged.
677 Columns represent the means ($n = 4$) \pm SE. Different letters mark significant differences ($P < 0.05$)
678 between means according to ANOVA after data normalisation. In frame b, $P < 0.001$.



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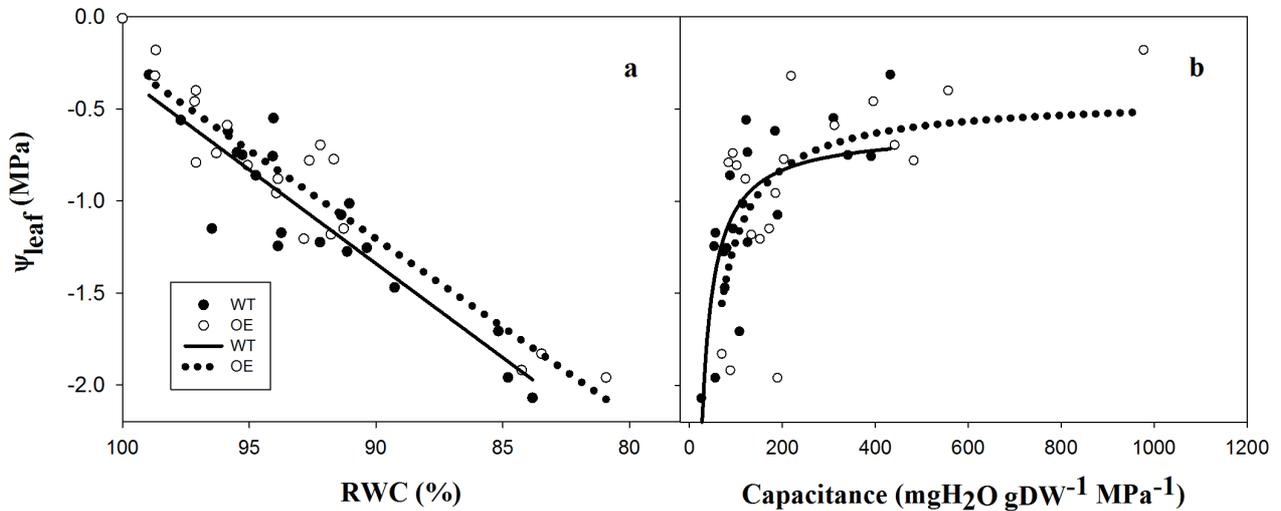
682 **Figure 6:** expression of endogenous and transgenic *PIP*-type AQP genes in WT and OE lines under
 683 dark and light transpirative conditions in rehydrated leaves. Relative expression levels of *VvPIP1;1*,
 684 *VvPIP2;1*, *VvPIP2;2*, *VvPIP2;3*, endogenous *VvPIP2;4N*, and transgenic *VvPIP2;4N* were
 685 determined by qRT-PCR in leaves. The PCR data were normalised with those for UBI transcripts.
 686 Data are expressed as the mean \pm SE; different letters denote significant differences at $P \leq 0.05$.



687

688

689 **Figure 7:** relationship between Ψ_{leaf} and RWC (a) and Ψ_{leaf} and C (b). Data were obtained from leaves
 690 allowed to dehydrate in darkness in the laboratory atmosphere. Filled circles represent the WT leaves; open
 691 circles represent OE leaves. Solid and dotted lines correspond to the regression of WT and OE, respectively.



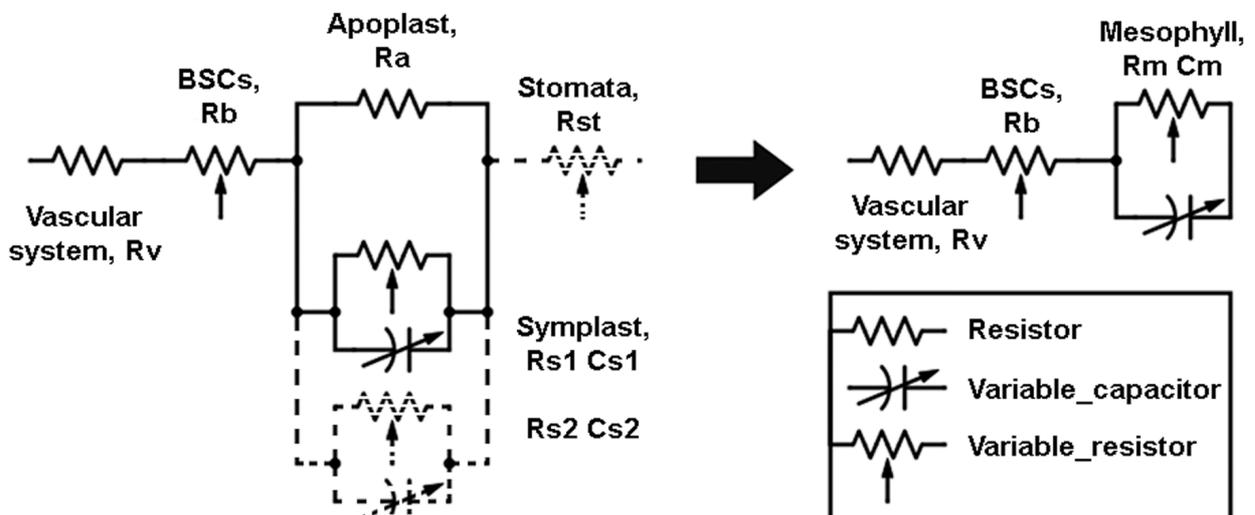
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694

695 **Figure 8:** schematic representation of the leaf hydraulic pathway. The leaf is divided in several
 696 compartments represented by resistors (vascular system, R_v ; Bundle sheath cells, BSCs R_b ; apoplast, R_a ;
 697 symplast R_s1 R_s2 ; and stomata R_{st}) and capacitors (symplast, C_s1 C_s2). Dashed lines indicate additional
 698 parts that can be added to the system. When transpiration is stopped, the system could be simplified by
 699 summing the capacitors and the reciprocal resistors (right side).

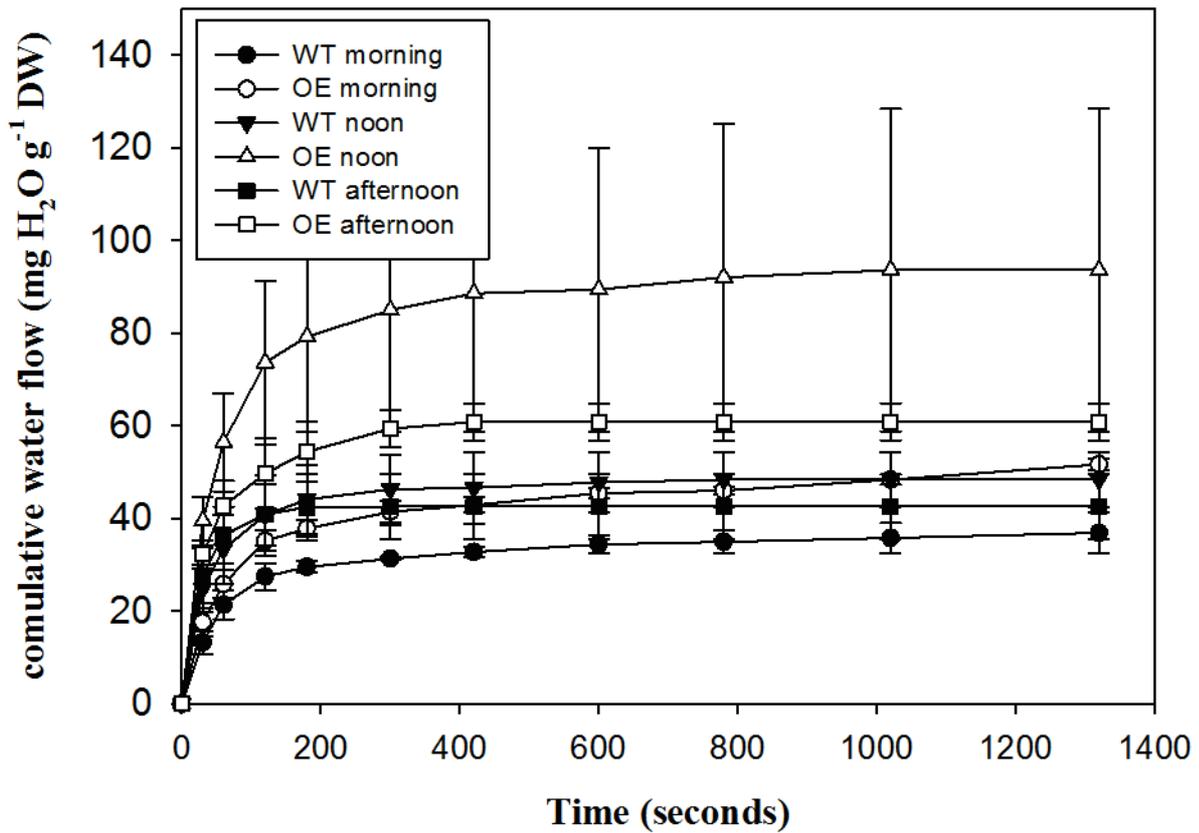
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701

702 **Figure S1:** time-course of the cumulative water flow out of leaves in WT leaves (filled symbols) and OE
703 leaves (empty symbols) in the +0.5 experiment. Data (corresponding to the ones in Fig. 2) were plotted
704 according to the time of the day when the experiment was performed: morning (8:00–10:00, circles), noon
705 (11:00–13:00, triangles) and afternoon (14:00–16:00, squares). Symbols represent the means \pm SE (n = 2).

706

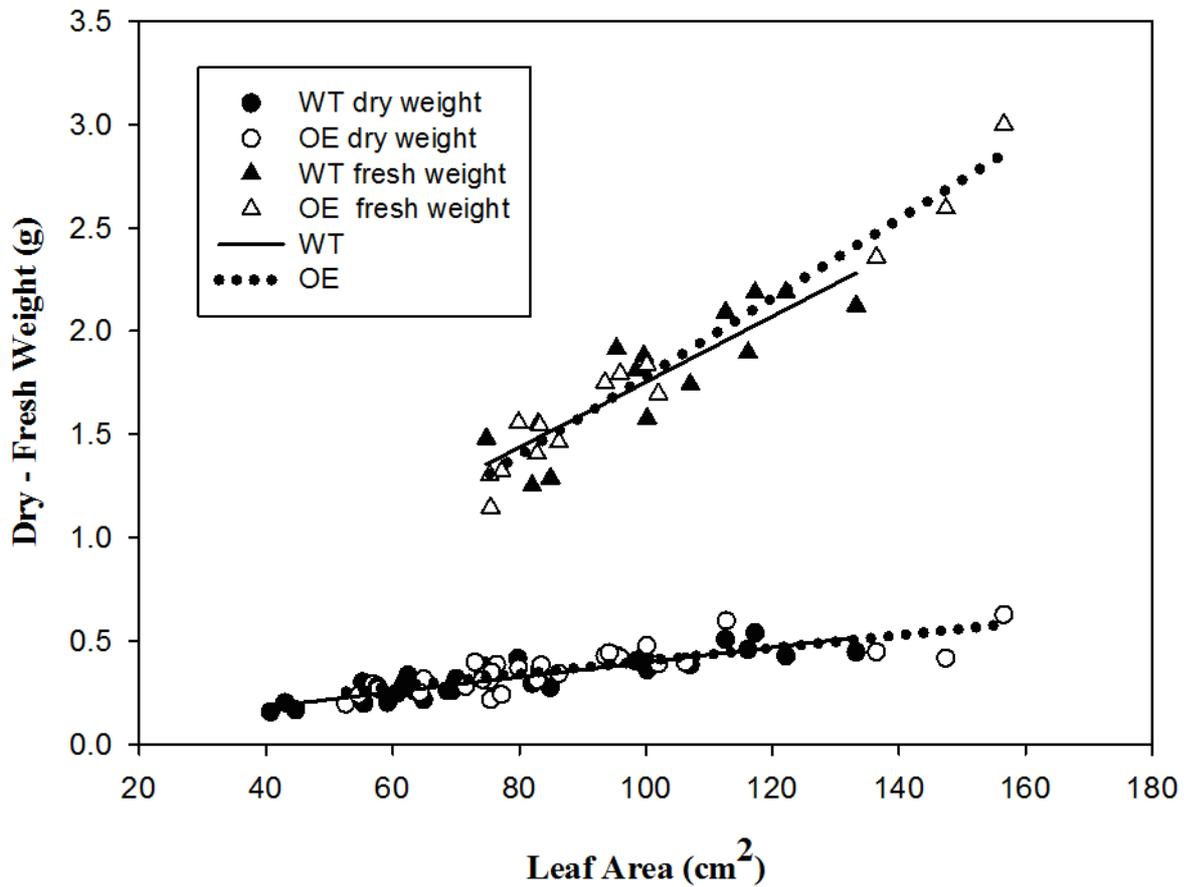


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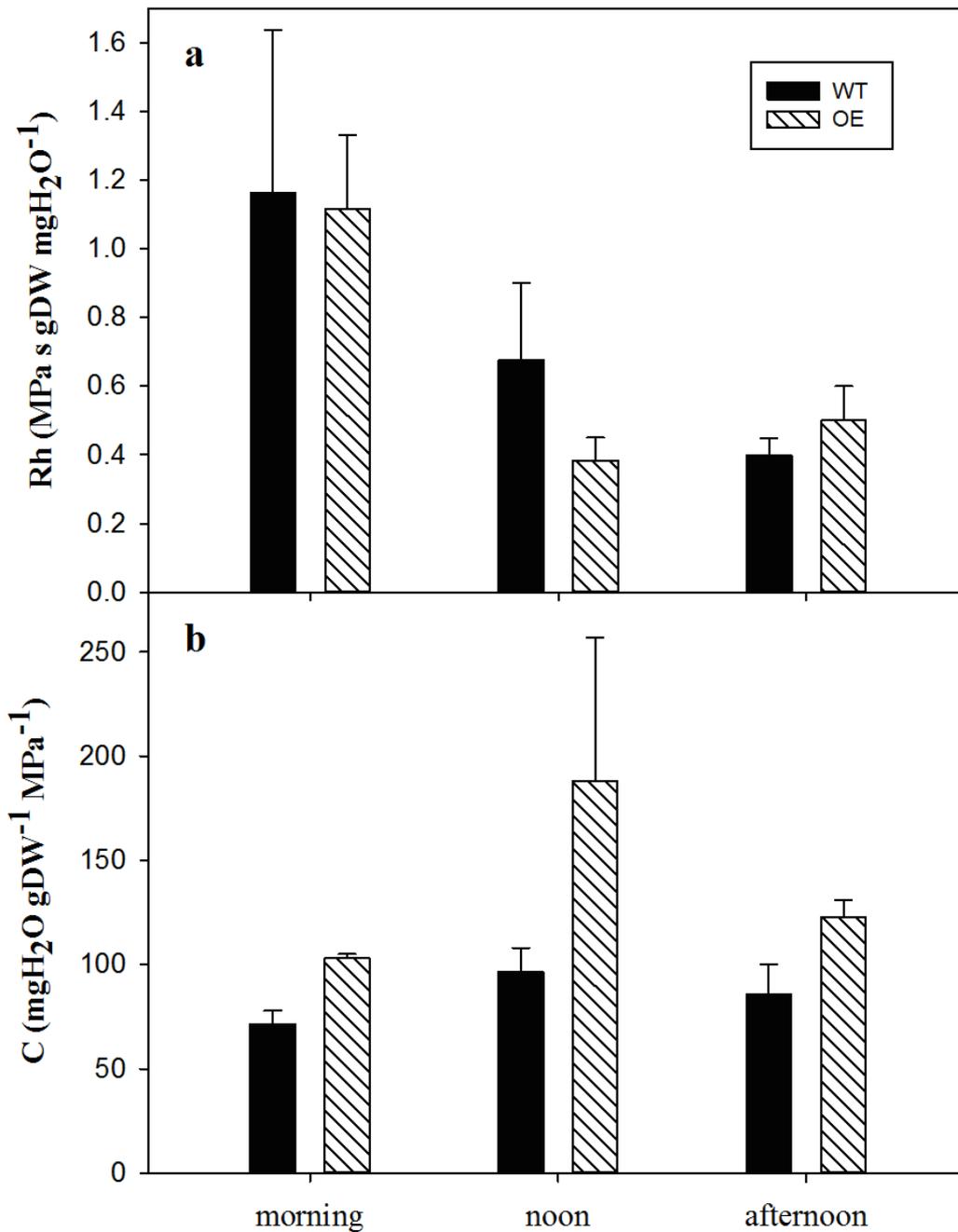
709

710 **Figure S2:** relationship between dry weight and leaf area (circles) and fresh weight and leaf area
711 (triangles) in WT (filled symbols) and OE leaves (empty symbols). Regression lines are shown for
712 WT (solid trend line) and OE (dotted trend line).



713
714
715

716 **Figure S3:** a) hydraulic resistance (Rh), and b) capacitance (C) in WT leaves (black columns) and
717 OE leaves (grey columns) obtained by pressurising the leaves to +0.5 MPa (+0.5). Columns
718 represent the means ($n = 2$) \pm SE. Means were obtained by averaging the measurements performed
719 at different times of day (morning 8:00–10.00; noon 11:00–13.00; afternoon 14:00–16.00 hours).
720 Averages were obtained from the same datasets of Fig. 2 and 3 (only +0.5 treatment) and S1.



721

722

723 **Reflection coefficient implication on hydraulic capacitance**

724 We provide here the theoretical framework demonstrating the relation between the bulk leaf reflection
725 coefficient s and the bulk leaf capacitance C . The demonstration is based on a reanalyze of the Pressure-
726 Volume curve theory (Tyree and Hamel 1972).

727 Whole leaf water potential Y is usually considered as the algebraic sum of the pressure turgor potential P and
728 the osmotic potential P :

729

730 $Y=P+P$ (s1)

731

732 However, this equation is correct only when the reflection coefficient is equal to one. If s less than unity
733 then:

734

735 $Y=P+sP$ (s2)

736

737 When leaf dehydrate, its relative water content (RWC) decreased and the total relative loss of water R is
738 equal to :

739

740 $R=1-RWC$ (s3)

741

742 Assuming a constant apoplasmic water content fraction (af), we can compute the relative water content loss
743 R_s of the symplasmic compartment as:

744

745 $R_s=R/(1-af)$ (s4)

746

747 R_s is equal to 0 when the leaf is fully turgid and equals to 1 when the symplasmic compartment is empty.

748 Leaf capacitance C is defined as:

749

750 $C=dR_s/dY$ (s5)

751

752 or, as a proxy, as:

753

754 $C=(R_s(Y_1) - R_s(Y_2)) / (Y_2 - Y_1)$ (s6)

755

756 We will focus here on our +0.5 experiment, where $Y_1 = 0$ and $Y_1 = -0.5\text{MPa}$

757

758 Defining P_0 as the osmotic potential at full leaf turgor and e as the bulk leaf modulus of elasticity, we can
759 express P and P as a function of R_s as:

760

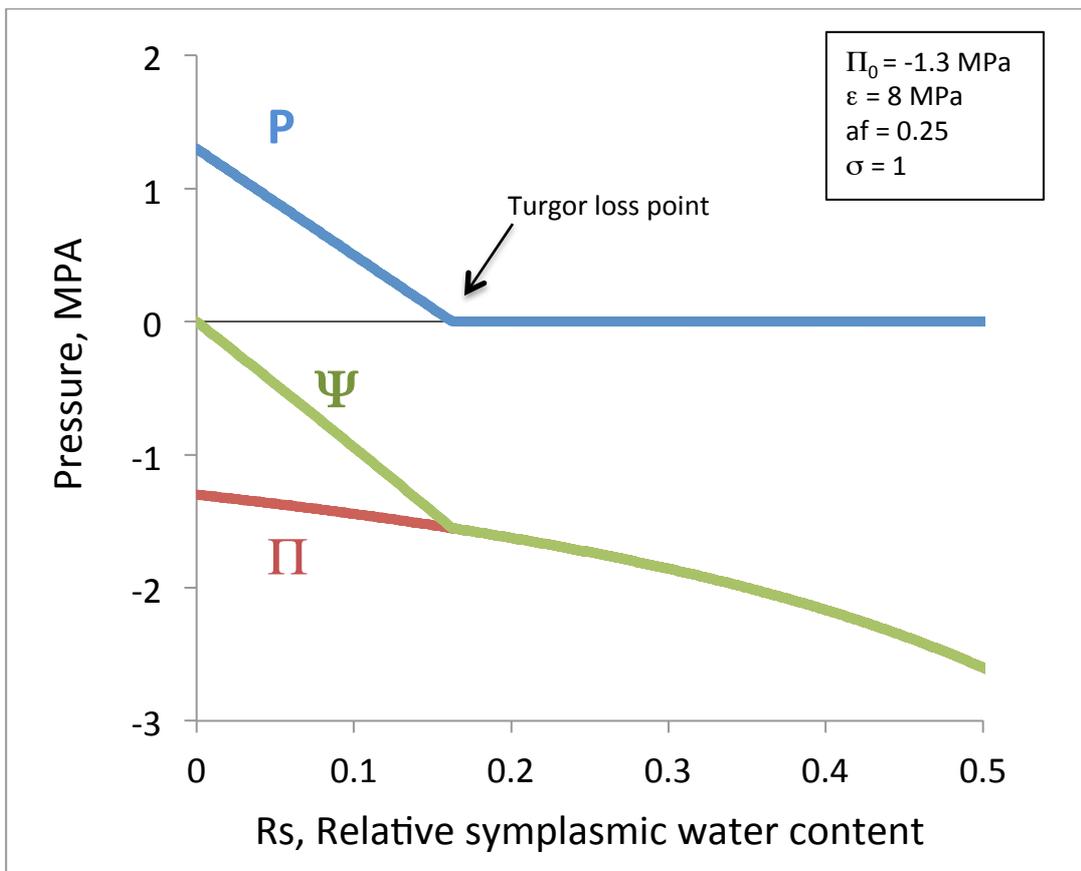
761 $P = -s(P_0 + eR_s); P > 0$ (s7)

762 $P = sP_0 / (1 - R_s)$ (s8)

763

764 Equations s1, s7 and s8 are used to construct a Höfler diagram (figure s1):

765



766

767

768 Figure S4: Höfler diagram showing the changes in whole leaf water potential (Y), pressure potential (P) and
769 osmotic potential (P) as a function of relative symplasmic water content. The parameters used to construct

770 the diagram are shown in the insert. These parameters were obtained on *Vitis* leaves similar to those used in
771 this study.

772 Combining s1, s7 and s8 we have:

773

$$774 \quad Y = -s(P_0 + eR_s) + sP_0 / (1 - R_s) \quad \text{for } P > 0 \quad (s9)$$

$$775 \quad Y = sP_0 / (1 - R_s) \quad \text{for } P = 0 \quad (s10)$$

776

777 By solving equations s9 and s10 we can express R_s as a function of Y as:

778

$$779 \quad R_s = \frac{\sigma(\varepsilon - \Pi_0) - \Psi - \sqrt{(\Psi + \sigma(\Pi_0 - \varepsilon))^2 + 4\sigma\varepsilon\Psi}}{2\sigma\varepsilon} \quad \text{for } P > 0 \quad (s11)$$

780

$$781 \quad R_s = 1 - sP_0/Y \quad \text{for } P = 0 \quad (s12)$$

782

783 Exact solutions of C can then be derived from s11 and s12 using s5 or s6.

784 A proxy of C can also be obtained if we assume that for low R_s values equation s8 can be approximated by:

785

$$786 \quad P \approx sP_0 \quad (s13)$$

787

788 then, by combining s2, s7 and s13 we have:

789

$$790 \quad Y \approx -seR_s \quad (s14)$$

791

792 then it comes:

793

794 $C \approx 1/se$ (s15)

795

796 The relative change of whole leaf capacitance C_{rel} when the reflection coefficient decreases from 1 to s is:

797

798 $C_{rel} \approx 1/s$ (s16)

799

800 The relations between C_{rel} derived from s11 and s16 and s are shown in figure s2. The approximation is
801 robust but valid only when R_s is low and s is high (>0.5).

802 Therefore, dividing s by two will double approximately the whole leaf capacitance and this effect is largely
803 independent of e , P_0 and Y .

804

805

806

807 Figure S5. Effect of the reflection coefficient s on the relative change in leaf capacitance. The exact relation
808 is shown in green and the approximation given in equation s16 is shown in red.

809