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► To cite this version:

Beatriz Muries Bosch, Robin Mom, Pierrick Benoit, Nicole Brunel-Michac, Hervé Cochard, et al.. Aquaporins and water control in drought-stressed poplar leaves: A glimpse into the extraxylem vascular territories. *Environmental and Experimental Botany*, 2019, 162, pp.25-37. 10.1016/j.envexpbot.2018.12.016 . hal-02168616

HAL Id: hal-02168616

<https://hal.science/hal-02168616>

Submitted on 22 Oct 2021

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6 **Aquaporins and water control in drought-stressed poplar leaves: a**
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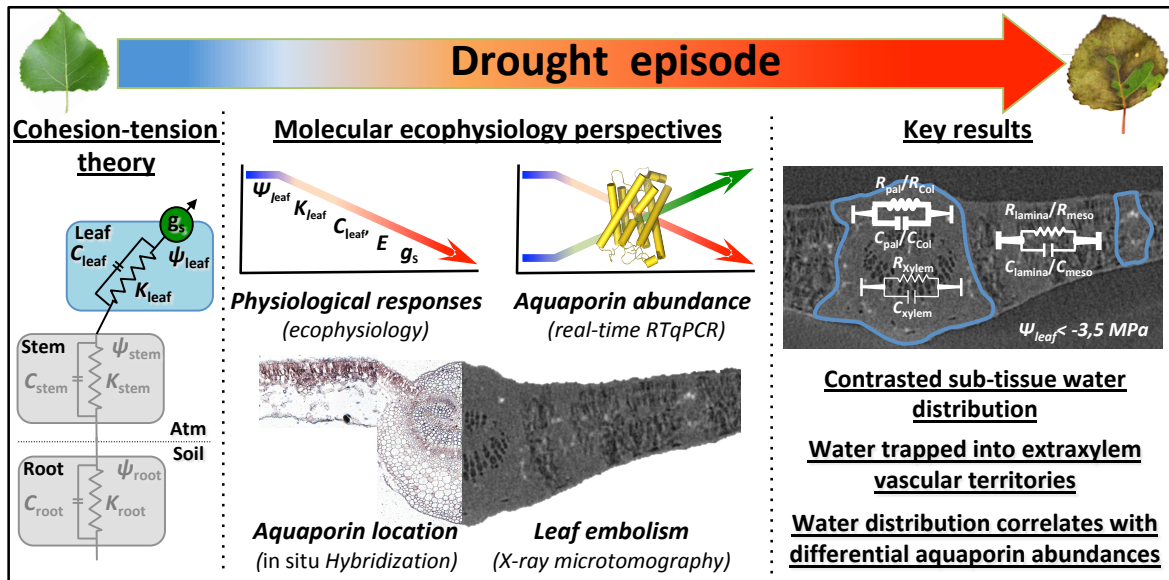
35 **Abstract**

36 Leaf hydraulic conductance (K_{leaf}) and capacitance (C_{leaf}) are among the key parameters in
37 plant-water regulation. Understanding the responses of these hydraulic traits to drought
38 conditions remains a challenge for describing comprehensive plant-water relationships. The
39 ability of an organism to resist and/or tolerate embolism events, which may occur at high
40 negative pressure caused by hydric stress, relies on how well it can sustain a hydraulic
41 system in a dynamic equilibrium. *Populus deltoides* is a water-saving tree species with a
42 stomatal conductance that declines rapidly with reduced water availability. Under unfavorable
43 conditions, the stomatal control of transpiration is known to be closely coordinated with a **loss**
44 **of plant hydraulic functioning** that can ultimately result in hydraulic failure through xylem
45 embolism, notably in leaves. The effects of drought on leaf hydraulics are also related to
46 regulation in water permeases such as the aquaporins. To describe the responses linked to
47 leaf hydraulics under severe drought and rewatering conditions, water-stressed poplars were
48 monitored daily on an ecophysiological and a molecular scale. A structural and expression
49 analysis on a set of aquaporins was carried out in parallel by *in situ* hybridization analysis
50 and quantitative PCR. In complement, water distribution in water-challenged leaves was
51 investigated using X-ray microtomography. A general depression of leaf hydraulic
52 conductance and relative water content occurred under drought, but was reversed when
53 plants were rewatered. More interestingly, (i) extreme leaf water deficiency led to marked
54 xylem and lamina embolism, but a degree of hydric integrity in the midrib extraxylem
55 territories and the bundle sheath of the minor veins was maintained, and (ii) the sub-tissue
56 water allocation correlated well with an over-accumulation of several *PIP* and *TIP*
57 aquaporins. Our multi-facet molecular ecophysiological approach revealed that leaves were
58 able to secure a certain level of hydric status, in particular in cell territories near the “living
59 ribs”, which provided rapid hydric adjustment responses once favorable conditions were
60 restored. These findings contribute to an integrated approach to leaf hydraulics, thus favoring
61 a better understanding of the cell mechanisms involved in tree vulnerability to climate
62 changes.

63

64 **KEY WORDS**

65 *Populus*, Leaf, Extraxylem territories, Bundle sheath cells, Aquaporins, Cavitation



66

67 **Highlights**

68 Poplar, an important woody crop is sensitive to water availability.

69 Soil water supply substantially affects various ecophysiological traits linked to plant hydraulic
70 status and aquaporin abundance.

71 When xylem is embolized, extraxylem vascular territories and minor veins stay fully hydrated
72 and can be considered as “capacitors”.

73 **These capacitors would act as water pools in the leaves, pivotal lever to drought tolerance
74 and recovery.**

75 Specific sub-tissue water allocation correlates with differential **PIP** and **TIP** aquaporin
76 abundances.

77 1. Introduction

78 Water movement throughout a plant depends not only on stomatal aperture, but also on the
79 interconnected dynamic hydraulic conductances from roots, stem and leaves (Sack and
80 Holbrook, 2006; Heinen et al., 2009). Water potential gradient caused by plant transpiration
81 is the main driving force of these water transfers. In the cohesion-tension theory,
82 transpiration into the atmosphere draws water from the soil along a root-stem-leaf continuum,
83 creating a gradient in water potential (Ψ_w). Tension in the vascular system can reach very
84 high levels, generating a thermodynamically highly metastable biophysical state. During
85 drought, low soil water content exacerbates this tension. As a result, the sap tensions can
86 exceed a threshold value above which vaporization can occur in the xylem conduit and
87 disrupt water columns in the lumen of xylem vessels, an effect called cavitation. The resulting
88 embolized conduits become non-functional for water supply. This process is widely believed
89 to be a substantial cause of the decline in plant hydraulic conductance that occurs during
90 drought. It can ultimately precipitate premature mortality of plant organs (including leaves),
91 and sometimes plant death (Choat et al., 2012). A very extensive and intricate pattern of
92 veins adorns leaves. Embolism events are theoretically expected to propagate between
93 interconnected conduits, suggesting that the leaf could be an organ highly vulnerable to
94 dehydration. However, *in vivo*, the network architecture plays a substantial role in the
95 differential propagation of embolism. Interestingly, a high gradient in vulnerabilities to
96 dehydration is observed and depends on the hierarchic level of venation: embolism
97 vulnerability increases proportionally with the size of veins, and the initial nucleation occurs in
98 the largest veins (Scoffoni and Jansen, 2016, Brodribb et al., 2016ab; Hochberg et al., 2107;
99 Scoffoni et al., 2017ab-2018). As leaves form a major hydraulic bottleneck impacting overall
100 plant hydraulic responses (Sack and Holbrook, 2006), deciphering the mechanisms of
101 embolism and their effects on hydraulic function in leaves is an essential adjunct to studies
102 on stems.

103 By its position within the canopy, the leaf system experiences widely fluctuating ambient
104 conditions such as irradiance and temperature, while depending on the hydric status of the
105 tree overall (Dobra et al., 2010). Most environmental stresses share common effects and
106 adaptive responses in leaf physiology, but they usually result in hydraulic disruptions,
107 cavitation in veins, and tissue dehydration in leaves. Dehydration status arises from an
108 imbalance between tissue water uptake or storage, and tissue reallocation or loss through
109 transpiration (Jackson et al., 2003). As an adaptive response, leaves rapidly close their
110 stomata to modulate stomatal conductance (g_s). However, transpiration is involved in carbon
111 uptake for photosynthesis and general metabolism, and in preventing heat damage in leaves
112 exposed to full sunlight. But for plant species that exhibit high sensitivities to drought stress,

113 impaired transpiration thus becomes hazardous in leaf tissues. Concomitantly, osmotic
114 adjustments can occur, involving the neo-synthesis and/or accumulation of small compatible
115 solutes (or osmolytes), e.g. proline, sugars, glycine betaine and some inorganic ions (Khan
116 et al., 2010). These entities help the cells maintain the structural integrity of membranes and
117 intrinsic molecular components that are in a dehydrated state. A barely explored hypothesis
118 is that dehydrated leaf tissues or cells can buffer variations in leaf water potentials by using
119 their own water storage: plant tissues can safeguard cell integrity (*i.e.* metabolism) by
120 buffering the effects of small fluctuations in water potential. By analogy with an electric circuit
121 (van den Honert, 1948), this property is termed hydric capacitance (Koide et al., 1989).

122 Physiologically, leaf water storage capacitance C_{leaf} measures the ability of a leaf to store
123 and reallocate water to buffer its own hydraulic status. Relative intrinsic capacitance is
124 defined as the mass of water that can be extracted, *i.e.* reallocated, per unit change in the
125 water potential of the tissue. By definition, capacitance fundamentally integrates the complex
126 water network of a tissue, working in parallel with the water transport efficiency (hydraulic
127 conductance, K_h) and in series with the absolute water storage capacity (or relative water
128 content, RWC) of the tissue. Despite its importance, leaf capacitance has been paradoxically
129 under-investigated.

130 The relevance of C_{leaf} is clearly evidenced in desert succulent plants, which are highly
131 persistent in water-limited environments. Interestingly, these species present a water
132 transport from the water-storing parenchyma to the photosynthesizing collenchyma (*a.k.a.*
133 "*chlrenchyma*"), displaying contrasting capacitance between these two distinct but
134 neighboring cell types: high C for water storage parenchyma cells and lower C for
135 chlrenchyma (Schmidt and Kaiser, 1987; Smith et al., 1987; Tissue et al., 1991; Vendramini
136 et al., 2002; Nobel, 2006). A high-capacitance water-storing parenchyma with stable water
137 potential gradients is thought to provide an efficient mechanism to ensure a supply of water
138 from its storage tissues that actively sustains photosynthesizing cells for longer in periods of
139 drought (Martin et al., 2004; Nobel, 2006). In temperate woody species without prominent
140 leaf water storage tissues, osmotic and hydrostatic pressures affect inter-tissue water
141 transfer and related capacitances. Even though C_{leaf} is a well-defined parameter, it is still
142 neglected when assessing the vulnerability of leaves to drought-induced hydraulic
143 dysfunction, "leaf water storage" being the preferred concept. Overall, it is assumed that
144 leaves with high K_{leaf} values and high maximum transpiration levels benefit most from stored
145 water to buffer fluctuations of available water (Aasamaa and Sober, 2001; Aasamaa et al.,
146 2001; Sack et al., 2003). There is a need for a better understanding of this hydraulic trait,
147 C_{leaf} , and especially of the underlying molecular mechanisms that govern it.

148 Leaves, and especially their veins, function as microfluidic circuitry. The hydraulic
149 resistances are not constant, but dynamic, and can vary nonlinearly with water potential. In
150 addition, this ecophysiological trait primarily concerns a living structure, the cell, which is
151 delimited by a semi-permeable membrane. Thus these dynamic water movements across
152 biological membranes are fundamental for maintaining proper fluid balance within and
153 between cells, and within different anatomic compartments (inner leaf tissues such as xylem
154 parenchyma, bundle sheaths and mesophyll cells). Models of dynamic hydraulic flow in
155 tissues and organs integrate aquaporins (AQP) as molecular actors in the regulation
156 mechanism (Sade and Moshelion, 2016).

157 AQP are membrane-spanning proteins that were assigned to the superfamily of the major
158 intrinsic proteins (MIP). They are involved in the specific transport of water and small neutral
159 solutes or dissolved gases across plasma and intracellular membranes (Prado and Maurel,
160 2013; Chaumont and Tyerman, 2014). These selective channels are present in all life forms,
161 whether mammals, amphibia, insects, plants, or bacteria (Heymann and Engel, 1999). In
162 angiosperms, several subfamilies of MIPs have been identified, typically clustered into five
163 main subfamilies with the plasma membrane intrinsic proteins (PIP), tonoplast intrinsic
164 proteins (TIP), nodulin-like intrinsic proteins (NIP), small basic intrinsic proteins (SIP) and the
165 non-exclusive X-intrinsic proteins (XIP) (Danielson and Johanson, 2008; Anderberg et al.,
166 2012). Plant AQPs show a remarkable diversity: *Populus* exhibits 54 AQP members from all
167 five subclasses (Gupta and Sankararamakrishnan, 2009; Lopez et al., 2012). Some of them
168 have been extensively investigated, and recent insights into their structural signatures,
169 expression patterns, subcellular localizations, and substrate specificities have led to models
170 that target the regulation of transport activity in various physiological plant processes. Yet
171 despite extensive research into aquaporin function and regulation for coordinated dynamics
172 of K_{leaf} or g_s (Heckwolf et al., 2011; Ben Baaziz et al., 2012; Ferrio et al., 2012; Lopez et al.,
173 2013), studies of their potential physiological roles in regulating leaf capacitance in plants
174 challenged by unfavorable drought conditions are still sparse (Vitali et al., 2016).

175 In this study, we designed an experiment in which a set of poplar trees experienced a
176 gradual controlled drought for several days, followed by rehydration. Based on leaf
177 ecophysiological response, we monitored the molecular response, focusing on the
178 contributions of PIP and TIP aquaporin subfamilies by analyzing the accumulation transcript
179 patterns of all related members and the spatial distribution of some of those most expressed.
180 We also investigated where the embolism events occurred in leaf veins during leaf
181 dehydration to turgor loss and beyond, using X-ray microtomography (microCT), which
182 allows direct visualization of embolized conduits and their anatomic characteristics in

183 different vein orders. We discuss our results in terms of ecophysiological, molecular and
184 structural findings.

185 2. Materials and Methods

186 2.1. Plant material and drought experimental design

187 Experiments were conducted on two-year-old *Populus* trees (*Populus deltoides*, Marsh)
188 cultivated in 20 liter pots filled with commercial substrate (40% black, 30% brown and 30%
189 blond peat moss, pH 6.1, DUMONA-RN 75-3851, Arandon, the Netherlands). Plants were
190 grown in a controlled-environment greenhouse (Clermont Auvergne University, Clermont-
191 Ferrand, France) under a 16 h light/8 h dark photoperiod at 18-22°C (night / day) and with
192 relative humidity set at 70±10%. When incoming sunlight in the greenhouse was below
193 350 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (dawn and dusk), photosynthetic photon flux was maintained using 400W
194 Master son-T Pia Hg-free lamps (Philips). Three weeks beforehand, and during the
195 experiments, the plants were placed in an open but controlled environment where day/night
196 temperatures were around 17°C and 26°C, respectively, and the PAR at midday were
197 around 1450 $\mu\text{mol m}^{-2} \text{s}^{-1}$; conditions are detailed in Supplemental Figures S1. Pots were
198 automatically watered by drip irrigation, maintaining daily field capacity. For the experiment,
199 three control plants were continuously well-watered (number of trees sufficient for a control
200 physiological situation because of the very homogeneous responsiveness of *Populus*
201 *deltoides*), and nine plants were drought-challenged by withholding irrigation (greater number
202 of trees to cover eventual heterogeneous responsiveness during stressful conditions). Once
203 the hydric stress was reached ($\Psi_{\text{leaf}} < -3,5 \text{ MPa}$), but before the earliest signs of leaf wilting
204 (for Ψ_{leaf} between -3,5 MPa and -4 MPa for *Populus deltoides* in our environmental
205 conditions), droughted plants were rewatered. During the rehydration phase, four kinetic
206 points were targeted: 12 h after rewatering (Day 9 of experimentation), two days after
207 rewatering (Day 10 of experimentation, which corresponded to no significant change in
208 ecophysiological traits), three days after rewatering (Day 11 of experimentation, which
209 corresponded to a significant early transpiration recovery) and ten days after rewatering (Day
210 18 of experimentation when the predawn $\Psi_{\text{leaf}} = 0.0 \text{ MPa}$, and when the transpiration rate
211 was fully recovered). The water stress conditions of seven plants (three control and four
212 stressed plants) were indirectly assessed through water loss by evapotranspiration by
213 measuring the micro-variations in stem diameters and the total mass of the experimental
214 system (Figure 1). The micro-variations in stem diameters were continuously measured
215 using linear variable displacement transducers (LVDTs) (LVDT model DF5, Solartron
216 Metrology, Massy, France) connected to a data acquisition system (CR3000 Micrologger,
217 Campbell Scientific, Inc., Logan, UT, USA). LVDTs were installed at a height of 10 cm from
218 the base of the trunk. Changes in diameter variation (accuracy < 1 μm) were logged at 10 s
219 intervals, and 5 min means were recorded. Experimental systems (*i.e.* pot and plant) were
220 continuously weighed to check water deficit by placing them on a calibrated scale (PCE-

221 BDM-15, readability from 0.1 g, PCE, France) connected to a data acquisition system
222 (CR1000, Campbell Scientific). For the ecophysiological and molecular analysis, fully
223 expanded leaves from the top of each tree were sampled.

224 2.2. Leaf water potentials and gas exchange parameters

225 Predawn (5:00) and midday (14:00) leaf water potentials (Ψ_{leaf}) were measured using a
226 Scholander pressure chamber (PMS Instrument Company, Model 1505 D, Albany, OR,
227 USA). Daily predawn and midday leaf stomatal conductance (g_s) and transpiration flux (E)
228 were measured with a portable photosynthesis measurement system (LI-1600, Li-Cor,
229 Lincoln, NE, USA) under ambient conditions. Leaf hydraulic conductance (K_{leaf}) was
230 assessed according to Attia et al. (2015), and calculated by using the equation $K_{\text{leaf}} = E /$
231 $(\Psi_{\text{stem}} - \Psi_{\text{leaf}})$. This approach uses the ratio of the transpiration flux (E) to the water potential
232 difference ($\Delta\Psi$) between the xylem at the leaf entry (Ψ_{stem}) and the bulk leaf (Ψ_{leaf}). E
233 measurements were made with the LI-COR portable photosynthesis system (Li-1600), and
234 Ψ_{leaf} was monitored immediately after with the Scholander pressure chamber. Predawn K_{leaf}
235 calculation (at 05:00) was enabled by non-zero predawn E values. Stem xylem water
236 potential (Ψ_{stem}) was estimated at 05:00am and 2:00pm by sealing three leaves for each
237 analysis in a plastic bag wrapped in aluminum foil in the late afternoon of the day before
238 measurement. Each Ψ_{stem} corresponds to the mean of the water potentials monitored from
239 these three leaves. Transpiration was thereby prevented, and water tension was promoted to
240 equilibrium at the point of attachment of the leaf to the stem (Begg and Turner, 1970). Leaf
241 area (m^2) was measured using an ImageJ macro after scanning. A Höfler diagram
242 determination is based on the pressure-volume curve theory according to Tyree and Hammel
243 (1972). To construct the Höfler diagram and plot the related PV curve, changes in water
244 potential and relative water content θ (%) = $[(FW - DW) / (TW - DW) \times 100]$ were monitored as
245 the tissue dehydrated (where FW, DW, and TW are the fresh, dry and turgid masses,
246 respectively). This relationship was quantified by allowing excised tissues (such as defoliated
247 leaves) to slowly and artificially desiccate on a laboratory bench during which time water
248 potentials and tissue weight were periodically measured. Leaf water storage capacitance per
249 unit leaf area and unit water content ($C_{\text{leaf}}; \Delta\theta / \Delta\Psi \cdot \text{s}^{-1}$) was obtained from these PV curves
250 according to the procedures of Sack et al. (2003), Sack and Pasquet-Kok (2011) and Scholz
251 et al. (2010).

252 2.3. Quantitative reverse transcription polymerase chain reaction analysis (RT-qPCR)

253 One leaf from three trees (which corresponds to three independent biological replicates) was
254 sampled at 5:00am and 2:00pm, at Days -1, 6, 7, 8, 9, 10, 11 and 18 of the experimentation.
255 Sampled leaves were depetiolated and immediately frozen in liquid nitrogen for subsequent
256 molecular analysis. For total RNA extraction, samples (25-100 mg) were ground to a fine

257 powder in liquid nitrogen and stored at -80°C until use. Total RNA was extracted according to
258 Chang et al. (1993) with some modifications. Briefly, leaf powders were transferred into 1 ml
259 of lysis CTAB extraction buffer (cetyltrimethylammonium bromide). The homogenate was
260 incubated for 5 min at 65°C and treated twice with 1 volume of chloroform:isoamyl alcohol
261 (24:1). The supernatant was collected and treated overnight in 2 M LiCl at -20°C. The
262 precipitate was collected by centrifugation (16,000 g for 45 min) and was washed with 70%
263 ethanol. The pellet was dissolved in 25 µl of water (DEPC), and treated with 10 U RNase-
264 free DNase (Promega, Madison, WI, U.S.A.) for 30 min. After two chloroform:isoamyl alcohol
265 (24:1) washes, total RNA was precipitated with 100% ethanol (2V) 2 h at -20°C. After
266 centrifugation at 16,000 g for 30 min, the pellet was washed with 70% ethanol, dissolved in
267 50 µl of water (DEPC) and stored at -80°C for further analysis. RNA concentrations were
268 determined by spectrophotometry (OD 260/280) (spectrophotometer ND-1000, Nanodrop,
269 France), and quality was checked using 2% TAE/agarose electrophoresis. First-strand cDNA
270 was synthesized from 2 µg of total RNA using the SuperScript® III First-Strand Synthesis
271 System for RT-PCR (Invitrogen™) and with oligo-dT, following the manufacturer's
272 instructions. The synthesized cDNA was diluted 10-fold with sterile water and used as a
273 template for PCR. The abundance of defense-related transcripts was determined by real-
274 time Q-PCR with an MyiQ instrument (Bio-Rad), using the comparative C_t method. PCR was
275 performed in a 96-well plate with a total volume of 15 µl, containing 10 µl of MESA GREEN
276 qPCR MasterMix Plus (Eurogentec), 0.5 µM (each) of forward and reverse primers, and 2 µl
277 of cDNA template. Amplifications were done using the following cycle settings: 94°C for
278 30 sec, followed by 35 cycles at 94°C for 15 sec, 54/58°C (according to primer pair) for
279 15 sec, 72°C for 20 sec. A dissociation curve was systematically generated to confirm the
280 amplification of the PCR single bands. The primer pairs used in this work were those from
281 Lopez et al. (2012). Each pair was verified *in silico* on the JGI genomic dataset (V12.1) from
282 *P. deltoides* (WV94, v2.1) concomitantly to molecular analysis on cDNA and gDNA samples.
283 The amplified fragments had an average size of 200 nucleotides. MIP nomenclature and
284 primers are listed in Supplemental Table S1. The differential state levels of gene expression
285 were calculated from the threshold cycle (C_T) and using the formula $2^{-\Delta\Delta C_T}$ according to Livak
286 and Schmittgen (2001). This corresponds to the fold change of an isoform at a given time
287 point relative to its expression from well-irrigated trees, or for daily assessment at their
288 related initial pre-dawn time points (5:00). For each biological sample, each PCR run was
289 performed in duplicate. Negative controls without cDNA were used in all the PCR reactions.
290 To normalize the amount of transcripts present in each reaction, five putative internal
291 controls [*Actin2*, *EF1α*, *SAND*, *TIP41-like* (for type 2A phosphatase activator TIP41), and the
292 *UP2* nomenclatures and primers given in Supplemental Table S1], were chosen from
293 different protein families to reduce the risk of co-regulation. These reference genes were

294 selected from a number of candidates using the software application BestKeeper (Pfaffl et
295 al., 2004), first to determine the most suitable reference genes from nine widely used
296 housekeeping genes (Czechowski et al., 2005; Xu et al., 2011), and then to estimate a
297 BestKeeper Index used as a calibrator. Lastly, signal intensity data for all transcribed PIP
298 and TIP genes were hierarchically clustered (Ward contrast, Euclidean distance), with R
299 (v3.4.3, R Core Team, 2017) and gplots (v3.0.1, Warnes et al., 2016) libraries.

300 2.4. *In situ hybridization*

301 Fresh leaves were harvested, cut and immediately fixed in 4% (w/v) paraformaldehyde +
302 1 µl/ml triton X-100 for 2 h at 4°C, and the fixation was then extended overnight in 4% PFA
303 alone. Fixed samples were rinsed in PBS (135 mM NaCl, 3 mM KCl, 1.5 mM KH₂PO₄, 8 mM
304 Na₂HPO₄, pH 7.2), then dehydrated and progressively embedded in paraffin (Paraplast,
305 Sigma Aldrich, Saint-Louis, MO, USA) (Brunel et al., 2002). Samples were cut into 10 µm
306 sections with a rotary microtome (HM340E, Microm International GmbH, Walldorf), mounted
307 on Superfrost Plus slides (Fisher Scientific, Elancourt, France) and dried at 42°C for one day
308 for *in situ* mRNA localization. Five gene-specific RNA probes were designed to be located in
309 the variable 3'UTR region of the *PdPIP1;1*, *PdPIP2;4*, *PdPIP2;10*, *PdTIP1;3* and *PdTIP2;1*
310 transcripts with an average size of 200 ribonucleotides (primers detailed in Supplemental
311 Table S1). DNA coding the probes was cloned in pGEM[®] T-Easy vector (Promega[®]). Sense
312 and antisense probes were synthesized by *in vitro* transcription as described earlier (Brunel
313 et al., 2002), and final detection was as described in Lopez et al., (2016). Observations were
314 made under an Axioplan 2 microscope (Zeiss, Jena, Germany). Data were recorded on a
315 digital camera (AxioCam HR, Zeiss) using Axiovision digital imaging software.

316 2.5. *3D X-ray microtomography observations*

317 Three-dimensional pictures of the internal structure of *Populus* leaves were acquired using
318 the X-ray microtomographic method (Nanotom 180 XS, GE, Wunstorf, Germany). A
319 minimum of two leaves were sampled at predawn (5:00 am) and midday (2:00 pm) from
320 control and stressed plants. Leaves were dipped in silicone oil to prevent severe dehydration
321 during sample preparation and image acquisition. Small disks were cut in the base of the
322 silicon oil-submerged leaf, at 5 mm from the petiole, including the midrib of the leaf. They
323 were placed in a gelatin capsule (diameter 5 mm and height 1.5 cm, size 4, Euromedex)
324 containing silicone oil. The field of view was ~2.5 × 2.5 × 2.5 mm³ and covered the full cross
325 section of the disks. X-ray settings were 60 kV and 240 µA. We used a molybdenum target to
326 increase contrast for low-density tissues. For each leaf, 1000 images (250 ms each) were
327 recorded during the 360° rotation of the sample. Full 3D volumes, with a final spatial
328 resolution of 2.5 × 2.5 × 2.5 µm³ per voxel, were reconstructed by Datos x 2.0 (Phoenix,

329 Nanotom 180 XS, GE, Wunstorf, Germany) software. Volumic image analysis and display,
330 including 2D slice extraction, were performed using VGStudio Max[®]2.1 software (Volume
331 Graphics, Heidelberg, Germany). After spatial calibration, leaf anatomy was examined by
332 image analysis using ImageJ software. Automatic segmentation enabled the isolation and
333 visualization of the embolized vessels. Indicative leaf water potential was estimated by the
334 mean of Ψ_{leaf} from the two leaves adjacent to those used for the microtomographic analyses,
335 using the Scholander pressure chamber described above. The water potentials indicated on
336 the figure 6AB were provided for information purposes only. Leaf shoots were not
337 equilibrated prior to measurement, generating values of leaf water potentials that can be
338 slightly overstated. Likewise, they do not reflect the water potentials of the disks.

339 2.6. Statistical analysis

340 Data for each biological assay are presented as the means \pm standard error (SE) of the
341 number of experiments (ecophysiological analyses, $n = 7$; molecular analyses, $n = 3$). All
342 quantitative data were processed with STATISTIX V8 software (2012) and using a one-way
343 analysis of variance (ANOVA) followed by a Tukey's honest significant difference (HSD)
344 *post hoc* test ($p < 0.05$). For the principal component analysis (PCA), ecophysiological and
345 molecular data were aggregated. Ecophysiological variables were Ψ_{leaf} , RWC, K_{leaf} , C_{leaf} , E
346 and g_s . Molecular data were qPCR expression levels for AQP. The PCA was performed
347 using R software (version 3.4.3, R-core Team, 2017) with FactoMineR (v1.39, Le et al.,
348 2008), missMDA (v1.11, Josse and Husson, 2016) and Factoextra (v1.0.5, Kassambara and
349 Mundt, 2017) libraries. Data were scaled to unit variance, and the number of dimensions for
350 the PCA was estimated at 10 by cross-validation.

351 3. Results and discussion

352 3.1. Drought impact on leaf hydraulics

353 Water deprivation is one of the most important factors responsible for down-regulating
354 an array of physiological processes in vascular plants. This down-regulation affects
355 transpiration status, which plays a central role in the systemic regulation of water streams,
356 creating a driving tension in plants from the root system. Our observations on leaf water
357 status, predawn and midday water potential in well-watered plants was -0.05 MPa and -
358 0.8 MPa, respectively (Figure 2A), matching those already reported in balsam poplar
359 (Larchevêque et al., 2011) and black poplar (Lopez et al., 2013). By contrast, in water-
360 depleted poplars, leaf water potential dropped remarkably to -2.6 MPa (Day 7) and beyond
361 (Day 8). These values **matched** a π_{tip} value at -1.67 MPa and a RWC_{TLP} of 86%
362 (Supplemental Figure S2) and confirm that *P. deltoides* is among those species with
363 moderate drought tolerance (Bartlett et al., 2012; Garavillon-Tournayre et al., 2017). In this
364 species, Tyree et al. (1992) showed a severe loss of conductivity **and** 100% embolism at
365 $\Psi_{xyl} = -2$ MPa. **As such, it was suggested that the eastern cottonwood (*P. deltoides*) is** among
366 those temperate species most vulnerable to cavitation. As a phenotypic trend characterizing
367 the *Populus* genus, this vulnerability relates to their riparian occurrence and their pioneer
368 species status (Fichot et al., 2015). There is a lack of data available on the leaf water
369 potential in *P. deltoides* under water stress in the field. For most studied species, when the
370 Ψ_{leaf} drops below -3 MPa, there is a loss of turgidity and/or death of all or part of the organ
371 (Blackman et al., 2009); this extreme is observable for *P. deltoides* when Ψ_{leaf} falls below -
372 4 MPa (data not shown).

373 By Day 6, Ψ_{leaf} started to decline significantly through water deprivation (Figure 2A).
374 Concomitantly, g_s and E started to decline, along with the closure of stomata (Figure 2EF).
375 **The tissue dehydration marked by a leaf water potential down to -3.5 MPa** (Day 8, Figure 2A)
376 was preceded by a gradual decrease in water flow underlined by drop in stomatal
377 conductance (Day 6, Figure 2F) and reduction of transpiration (Day 6, Figure 2E) as already
378 reported by Smith et al. (1987). Stomatal closure at early dehydration stages would reduce
379 tensions in the transpiration stream, maintaining a minimum **plant water potential** level that
380 prevents cavitation into the plant vascular system, from roots to leaves. In leaves, several
381 environmental signals (e.g. low CO_2 , high vapor pressure deficit) can modulate the stomatal
382 closure in relation with the early onset of drying (Bunce, 2006). However, the regulating
383 mechanisms of stomatal closure during dehydration can be questioned given reported
384 uncoupling between turgor loss and subsequent xylem cavitation (Bartlett et al., 2016;
385 Hochberg et al., 2017; Martin-StPaul et al., 2017).

386 In our work, even before leaves died, the fact that the leaf water potential at turgor
387 loss point (-1.67 MPa) corresponds to a RWC of 86% (that is 14% of leaf dehydration; Day 7;
388 figure 2AB; Supplemental figure S2) and that this leaf water potential rapidly declined further
389 to below -3,5 MPa for 35% dehydration (Day 8), is an important piece of the leaf homeohydr
390 puzzle. The indication that leaves may express safety mechanisms through leaf water
391 storage capacitance is an open question we address in this paper. Generally, leaves from
392 woody plants are not considered as water-storing organs. However, the lack of Ψ_{leaf}
393 decrease at Day 6 (Figure 2A) could be explained by the release of “stored” water from some
394 leaf tissues that remain to be identified (e.g. apoplastic compartment, symplastic network
395 from vacuoles, xylemic raw sap flow).

396 Similarly, possible water redistribution within the lamina may play a substantial role in
397 maintaining stress responsiveness and the integrity of the global metabolism. This protective
398 effect was observed primarily at Day 6 of drought, when g_s , E and K_{leaf} began to decline
399 (Figures 2FEC), while Ψ_{leaf} , RWC and C_{leaf} (Figures 2ABD) remained significantly stable.
400 Once Ψ_{leaf} dropped to around -1.5 MPa, C_{leaf} increased significantly until the turgor loss point
401 was exceeded (-1.67 MPa). It returned to the steady state before stress initiation. These
402 ecophysiological responses reflect, therefore, what we should expect from C_{leaf} . Although
403 activity of certain cellular processes starts to be modulated (or even impaired), C_h could
404 maintain acceptable relative water content in organs to slow down entry into turgor loss
405 (which results in a systemic risk of dehydration symptoms) thereby prolonging the
406 physiological activity of the organs.

407 The last ecophysiological aspects that need to be studied in correlation with previous leaf
408 traits are the stem diameter variations and the full mass of our experimental system (pot and
409 plant), which together reveal fluctuations in the general plant water status (Steppe et al.,
410 2006; De Schepper et al., 2012). Two significant fluctuations were analyzed: first the diurnal
411 variations in the control plants consecutive to depletion and refilling of internal stem water
412 storage, and second the behavior of plants in a progressive drought process, where the stem
413 diameters and the plant weight consistently dropped with no possible replenishment at night
414 to ensure tissue rehydration (Figure 1). In detail, the water content of the experimental
415 system brutally decreased in an irregular manner, then gradually, after Day 4. This biphasic
416 decline suggests evapotranspiration, followed by residual cuticle transpiration; two
417 physiological processes that act in concert for controlling plant water status and helps plants
418 survive under drought (Javelle et al., 2011). Stem diameters only broke down substantially
419 after Day 6 of drought, and significantly correlate with the onset of K_{leaf} , g_s and E decreases.

420 To complete this ecophysiological study, during rewatering, Ψ_{leaf} and RWC reverted
421 faster than g_s , E and K_{leaf} to pre-drought hydration levels. This slow recovery of both g_s - E and

422 hydraulic conductance, when the organs seemed to have secured renewable contents of
423 water pools, could be promoted by an overall gradual hydraulic repair.

424 **3.2. Expression of *PIP* and *TIP* in drought-challenged leaves**

425 It is now widely accepted that the aquaporins (AQP), ubiquitous pore-forming integral
426 membrane proteins, play an essential role in plant-water relations (Prado and Maurel, 2013).
427 In several plant species, multiple AQP isoforms are present ubiquitously in leaf tissues, and
428 their regulation under drought stress and rewatering, notably transcriptional, has pointed to
429 their involvement in leaf hydraulics (Galmés et al., 2007; Pou et al., 2013). However,
430 although the physiological roles of aquaporins in plants have been long debated, many
431 questions remains unanswered. We sought to gain a better understanding of how plasma
432 membrane and tonoplast intrinsic protein genes (*i.e.* *PIP* and *TIP*, respectively) in drought-
433 challenged *Populus* leaves are modulated, and to what extent these channels are linked to
434 the leaf-water relation.

435 The *Populus* genome contains 15 PIP members: 5 *PIP1*, 10 *PIP2*, and 17 *TIP* (Gupta
436 and Sankararamakrishnan 2009, Lopez et al., 2012). Based on hierarchical clustering
437 (Figure 3), nearly two-thirds of *PIP* and *TIP* genes (3 *PIP1*, 8 *PIP2* and 12 *TIP*) were
438 constitutively expressed and exhibited differential expression during stress. All of them
439 significantly exhibited differential two-phase responses between water deprivation and
440 recovery (Figure 3 and Supplemental Figures S3-S4). These contrasting expression levels
441 support the function assignment of these isoforms to leaf transcellular water flow.
442 Interestingly, up- and down-regulations occurred in isoform-balanced proportions. Down-
443 regulations concerned *PdPIP1;2-3*, *PdPIP2;6-7*, *PdTIP1;2-5-6-7*, *PdTIP2;1-2*, *PdTIP3;1* and
444 *PdTIP4;1*, and up-regulations concerned *PdPIP1-1*, *PdPIP2;1-2-3-4-10*, *PdTIP1;1-3* and
445 *PdTIP3;2*. Similar modulation of PIP and TIP expression levels during water stress and
446 recovery has been observed in many woody plants such as walnut, grapevine and *Populus*
447 (Sakr et al., 2003; Pou et al., 2013; Secchi et al., 2010; Lopez et al., 2013; Laur and Hacke
448 2014). Furthermore, the specific expression of *PdPIP2;2* (*PdPIP2;3* and *PdTIP3;2*, to a
449 lesser extent) during recovery suggests that poplar leaves sense rehydration quickly. These
450 molecular findings strengthen the view that recovery from stress should be seen as a specific
451 process and not simply a passive return to the initial steady state.

452 It is commonly accepted that aquaporins allow transmembrane water movement
453 along osmotic or hydraulic gradients. The fact that a set of AQP is up-regulated during water
454 deprivation suggests that leaves keep a constant fine-tuned balance between water loss and
455 water uptake. However, because drought impedes water uptake, we speculate that a
456 controlled adjustment of the whole AQP pool may occur differentially in various cell/leaf sub-
457 compartments and limit a catastrophic process of tissue death. It is thus of interest to know

458 how each AQP is involved in adjusting dynamic leaf hydraulic trade-off between main use
459 and storage capacities.

460 At this stage, we are unable to propose any functional interpretations of AQP isoform
461 steady state levels. The role of the AQP in the regulation of physiological status under hydric
462 constraint is highly complex and still largely unraveled, especially for plant species that share
463 important multigenic families. *Populus*, with 54 AQP (including 15 PIP and 17 TIP), is one
464 such (Secchi et al., 2009; Lopez et al., 2012). The contrasting patterns of expressed isoforms
465 suggest compensating events between isoforms, *i.e.* one AQP group taking over the
466 functions of the isoforms from another group, thus creating differential flow and possibly
467 preferential orientation of water flow. Similarly, the contrasting pattern of isoforms within
468 different AQP sub-families, whichever their expression group (up or down), reveals a
469 complex participation of aquaporins in water flow between cytosol and morphoplasm. They
470 could act in different phases of water deficit and recovery, as for *PdPIP2;4-10* or *PdPIP2;2*,
471 respectively. Unfortunately, the use of transgenic plants or specific mutants cannot provide a
472 clear answer in *Populus*, precisely because such a compensational effect could potentially
473 be produced by several endogenous AQP counterparts.

474 The AQP expression patterns reveal that their contribution to flow may be relatively
475 complex and highly regulated. To examine the collinearity between transcribed AQP, and
476 between transcribed AQP and ecophysiology variables, a principal component analysis
477 (PCA) was carried out (Figure 4). The first dimension (Dim1) and the second dimension
478 (Dim2) summarized 44.81% and 19.32% respectively of the global variance in the molecular
479 and ecophysiology response curves. Overall, AQP collinearized well with each other and with
480 ecophysiological responses, with the notable exception of C_{leaf} . In addition, the PCA analysis
481 revealed two AQP expression profiles, namely up- or down-regulated (Figure 3). In addition,
482 this PCA offers a useful glimpse of an AQP split that could be detected from hierarchical
483 plotting (Figure 3): one AQP group is essentially made up of TIP subfamily members
484 remarkably correlated with two ecophysiological parameters (g_s-E-K_{leaf} and $RWC-\Psi_{leaf}$) (on
485 the right part of the figure), and the second of several PIP subfamily members (plus
486 *PdTIP11*) that are clearly anticorrelated to RWC and Ψ_{leaf} (on the left of the figure). There are
487 no previous reports of this balance between TIP and PIP subfamilies during water stress in
488 *Populus*. Regulation of water status is a complicated process, in which PIP and TIP
489 aquaporins can account for the major proportion of the hydraulic conductivity of the plasma
490 membrane and tonoplast, respectively. PIPs take part in a transcellular water transport,
491 whereas TIPs take part in the regulation of water exchange between the cytosolic and
492 vacuolar compartments. During any cell environmental changes, TIP are involved in the fast
493 osmotic adjustments of the cytoplasm, then maintaining general osmolality and cell turgor.

494 Therefore, a fine balance between PIP and TIP subfamilies occurs, where the tonoplast
495 water permeability enables rapid osmotic adjustment in liaison with constant changes in
496 transcellular water flow, where PIPs also fulfill an essential complementary role.

497 Unfortunately, we cannot reasonably speculate on a comparative functional analysis within
498 and between MIP subgroups: TIP and PIP nomenclature is merely historical and does not
499 accurately reflect cell sub-localization, *i.e.* by definition TIP for tonoplast (vacuolar)
500 membrane or PIP for plasma membrane, respectively, knowing that PIP and TIP could be
501 inserted in both these membranes (Gattolin et al., 2011). That reflects a more complex and
502 fine-tuned regulation of aquaporin localization and activity in different physiological contexts.
503 Also, TIP are under-represented in studies of water flow through droughted tissues
504 compared with PIP, so that many questions about this subfamily remain unanswered. The
505 relative importance attached to PIP derives from a presumed dominant role conferred on the
506 plasma membrane in the transcellular pathway of water flow. The main reason for this is that
507 the hydraulic conductivity of the cell membrane is lower than that of the tonoplast membrane
508 (Maurel et al., 1997). Plant cells differ notably from other organisms (*e.g.* fungi, animals or
509 bacteria) in that intracellular spaces are dominated by a central large vacuole; these
510 differential water conductivities could therefore result in a fine-tuned control of water flow
511 from tonoplast to cytosol that minimizes any changes in cytosolic volume during water stress.
512 Previous studies have reported that several differentially expressed AQP genes seem to
513 have a strong impact on organ hydraulics (Chaumont and Tyerman, 2014). Here, PIP and
514 TIP displayed interesting, contrasting correlations that support the general assumption that
515 all these regulations imply assigned functions of several AQP isoforms from various
516 subfamilies. In addition, we assume that behind an apparent isoform abundance from this
517 MIP superfamily, a regulatory framework between counterparts should occur, notably co-
518 expressions with plausible hetero/homo-di/tetramerization events that would potentiate the
519 regulation of their transport activities (Fetter et al, 2004; Zelazny et al., 2007; Secchi and
520 Zwieniecki, 2010). Examining the AQP tissue localization will provide additional information
521 to decipher this intricate regulation network.

522 In previous work, authors report that most of the AQP show a relatively ubiquitous
523 expression in *Populus* (Gupta and Sankararamakrishnan, 2009), but to our knowledge, little
524 information has specifically revealed AQP subtissular distribution in leaves and differential
525 state levels of their expression. *In situ* hybridization (ISH) gave new insights that could help
526 in this respect. Among the 23 transcribed AQPs, we present the localization of five isoforms
527 (Figure 5; Supplemental Figures S5-S6). Interestingly, while *PdPIP2;10*, *PdTIP1;3* seemed to
528 show a rather widespread distribution in leaves, *PdPIP1;1*, *PdPIP2;4* and *PdTIP2;1* exhibited
529 a relative stronger expression in vascular bundles (mainly phloem) and collenchyma cells of

530 the midrib region. Furthermore, *PdPIP2;4* and *PdPIP2;10* showed a more pronounced
531 transcript abundance in the bundle sheath cells (BSCs), a layer of parenchyma cells
532 surrounding the vasculature particularly visible from the second-order veins in *P. deltoides*,
533 when staining for *PdTIP2;1* was confined essentially to cells nearest the vascular system and
534 was near-absent for *PdTIP1;3* in the whole of the BSC territory. These findings are highly
535 significant because these mRNA over-accumulations in AQP, which are more predominant in
536 all peripheral extraxylar vein tissues, are in line with the functional role of the extraxylary
537 veins and BSCs in the regulation of the leaf radial transport of water by acting as a xylem-
538 mesophyll hydraulic barrier, a mechanism that implies AQP activity (Sade et al., 2014; Prado
539 et al., 2013).

540 But what is probably even more interesting would be to integrate AQP in a complex of
541 integral membrane proteins that acts in the cell sensing. In drought, cells experience turgor
542 loss with an induced membrane shrinkage that could activate mechano- hydro- and osmo-
543 sensing channels or proteins: in mammals, AQP5 has been shown to interact with volume-
544 sensitive ion channels, indicating AQP involvement in cell volume sensing (Liu et al., 2006).
545 In water challenged leaves, and particularly in the mesophyll cells and the bundle sheath
546 cells, we could expect that differential threshold water potentials could trigger specific
547 hydrostimulus responses, which are still not understood. Could the protective responses of
548 capacitance then be a compromise between hydromechanical stimulus and physiological
549 influences where AQP and various partners act in concert?

550 Highly challenging areas for research are thus highlighted here: the spectrum of specific MIP
551 subfamilies involved in the physiological leaf responses to depletion and refilling of internal
552 stem water storage as well as their potential functional sub-compartmentalization in the
553 leaves remain to be elucidated.

554 **3.3. Water storage, extraxylem territories and AQPs**

555 The extraxylem pathway, and in particular the bundle sheath cells (BSCs), appears to play a
556 special role in regulating leaf water movements from vasculature to epidermis (Buckley et al.,
557 2015; Caringella et al., 2015; Ohtsuka et al., 2017). Furthermore, it is reported that AQP
558 regulate the water flow across cell membranes (Heinen et al., 2009; Shatil-Cohen et al.,
559 2011; Laur and Hacke 2014). Yet there is still a lack of knowledge on leaf water storage,
560 particularly in woody perennial organisms such as *Populus*. Micro-CT imaging provided
561 detailed spatial information about leaf sub-structure and related hydric status: most water-
562 and gas-filled conduits (phloem, xylem) and parachymal areas *lato sensu* (veins and lamina)
563 were clearly distinguishable, appearing filled or empty (light or dark gray, respectively). In the
564 present study, the level of embolized vessels is only qualitatively discussed because a

565 quantitative evaluation of this phenomenon remains uncertain on excision of small organs
566 that are under strong hydraulic tensions and there is always a risk of overestimating the
567 xylem embolism in the samples (Wheeler et al., 2013). Similarly, we cannot monitor the
568 hydration level of the different leaf tissues. Lastly, the water potentials displayed on the figure
569 6AB are only indicative and correspond to the mean of the Ψ_{leaf} from the leaves adjacent to
570 those used for the microtomographic analyses.

571 However, our analysis showed a remarkably contrasting sub-tissular distribution of the water
572 contents within a severely water-challenged leaf ($\Psi_{\text{leaf}} < -2.5$ MPa) compared with a well-
573 hydrated one (Figure 6AB). Xylem vessels appear highly embolized, and the spongy
574 mesophyll tissue in lamina appears to be dried out (cell shrinkage events resulting from
575 possible cell necrosis and/or intercellular spaces being emptied of water). That contrasts with
576 the cell structure in the leaf peripheral extraxylar vein tissues that seems to exhibit any
577 significant variation; this applies to all living tissues, *i.e.* phloem, sclerenchyma, parenchyma
578 and collenchyma cells. It is reasonable to assume that as any leaf tissues under severe
579 drought constraint, these territories undergo the decreasing phases of water status.
580 However, they exhibit relatively strong water retention efficiency. But even more interesting,
581 the analysis of these territories draws special attention to BSC. In many dicotyledons,
582 including *Populus*, the bundle sheath of secondary and minor veins extends towards the
583 epidermis forming a bundle sheath extension (BSE) that separates the leaf into specialized
584 compartments. It has been suggested that such leaves, referred to as heterobaric leaves, are
585 an adaptation for retaining water and protecting the mesophyll against drought stress
586 (Terashima, 1992). Bulk mesophyll cells only seems to suffer slight changes in water
587 potential and influence the dynamics of K_{leaf} in response to irradiance and leaf water status
588 (Sommerville et al., 2012; Sack and Scoffoni, 2013), as well as to stomatal control (Rockwell
589 and Holbrook, 2017). However, incorporating BSC in a protective scheme against overall leaf
590 dehydration as previously suggested by Griffiths et al. (2013) is still not simple as it is now
591 increasingly accepted that there is relatively little dehydration of this tissue during an extreme
592 drought. These BSC may be potential water stores or capacitors that are elicited in much
593 more extreme drought conditions. The paradox is that leaves are already at the limit of
594 hydraulic dysfunction, and have, therefore, gone into survival mode.

595 Tissue shrinkage and xylem embolism depend on the water potential of a plant challenged
596 by multiple environmental contexts. Physiologically, a sustained period of drought is highly
597 hazardous for plant integrity in general, but even more so for leaves because of their
598 structure and position on trees. Leaves endure continuous changes in their hydric status,
599 which conditions cell metabolic activity. In the event of drastic decline in leaf water potential,
600 general metabolic performance depresses and there is an considerable emergence of
601 “waterless holes” throughout the spongy mesophyll (major leaf metabolic scene).

602 Admittedly, leaves are the seat of photosynthesis, one of the most remarkable
603 bioengineering processes in the whole living world in which light energy is used to make
604 sugar. And so viewing leaves from deciduous trees as possible water capacitors is
605 uncommon, and therefore not a trivial matter. Yet, at the tissue level, persistent water
606 contents support the hypothesis that veins could act as a water storage network. However, if
607 a certain volume of water can be stored in veins (and possibly in BSC), how might the leaves
608 then benefit from it? Likewise, what are the regulatory mechanisms of the cell that enable
609 them to take water from the immediate cell environment, store it in possibly unfavorable
610 contexts of **water potential** and, if necessary, redistribute it in some way and that, to what
611 extent (the essence of all storage)? These issues obviously require further attention.

612 The aquaporins might offer one way to help address these questions. In a context where the
613 BSEs radial apoplast pathway of water and the passive water diffusion through cell
614 membranes are near-negligible, the water reallocations and/or retentions that occur by
615 expected modulation of the hydraulic resistance of cells have to be acutely controlled. In this
616 context, along with additional and modulated molecular events such as suberin and lignin
617 depositions in cell walls (Mertz and Brutnell, 2014; Ohtsuka et al., 2017) or osmolyte
618 accumulations (Williams et al., 1993), there is a strong presumption that leaf aquaporins are
619 involved in the regulation of the water supply from the xylem to the peripheral extraxylar
620 veins towards all of the extravascular compartments and the subsequent limb (Sack and
621 Holbrook, 2006; Lee et al., 2009; Shatil-Cohen et al., 2011). ISH suggests a sequential
622 allocation of different AQP members as the relay between the various leaf sub-territories,
623 which ensure a compartmentalized water distribution (Figure 5). Suitable modulations of
624 AQPs could thus co-design the hydraulic network architecture of transient water sources (or
625 reservoirs such as the peripheral extraxylar area) and sinks (lamina), and thereby, be the
626 trade-off between the capacitance and conductance processes. This hydraulic argument is
627 strengthened by the joint examination of X-ray microtomography (Figure 6AB) and ISH
628 outcomes (Figure 5), two complementary methodologies.

629 To our knowledge, this is the first time this biological demonstration has been
630 provided clearly. Figure 6C presents a schematic diagram, based on the electric circuit
631 analogy, showing the hydraulic model of water flow in leaves. It depicts the relationships
632 between the hydraulic resistances (R) and the AQP (one of the molecular mechanisms of
633 this resistance), and the hydraulic capacitances (C), which regulate the balance in water
634 diffusion and storage between leaf sub-territories.

635 **4. Conclusion**

636 Although a small fraction of daily transpiration is generally attributed to water stored in
637 leaves, the present study reveals the existence of several water storage compartments that

638 could act as water potential buffers, damping variations in leaf xylem. This phenomenon
639 pertains to the phloem and all the peripheral networks within the veins. The protective effect
640 may be regulated by the activity of aquaporins localized in the surrounding regions of leaf
641 vascular bundles, as some AQP members are differentially expressed with a contrasting
642 tissue-dependent pattern under drought and recovery conditions. Knowing the contribution of
643 each isoform in each leaf reservoir would provide more information about the regulation of
644 leaf hydraulic conductivity and capacity along the radial xylem-to-mesophyll pathway and
645 *vice versa* as a water-saving and drought-escaping mechanism.

646 Despite the volume of studies on leaf responses to water stress, many unanswered
647 questions linger. The diversity of the underlying mechanisms (*e.g.* ecophysiological,
648 anatomic, chemical and molecular) involved in the regulation of the leaf turgor maintenance
649 makes fully understanding water regulation by its molecular actors even more of a challenge.
650 These molecular ecophysiological findings highlight original observations, and further
651 research will now be needed to unravel the biological significance and the regulation levels of
652 the stress-strength-dependent dynamic water movement and storage observed in *Populus*
653 leaves.

654 **ACKNOWLEDGEMENTS**

655 This work was supported by the European Community's Seventh Framework Program
656 [FP7/2007-2013; FP7-PEOPLE-2011-IEF / proposal No. 303059]. We are grateful to two
657 anonymous reviewers for improving the manuscript.

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660

661 **AUTHOR CONTRIBUTIONS STATEMENT**

662 Beatriz MURIES co-designed and participated in all the experiments, and wrote the first draft
663 of the article;

664 Robin MOM, Pierrick BENOIT participated in the field experiments, and acquisition of
665 ecophysiological data;

666 Nicole BRUNEL-MICHAC supervised ISH experiments;

667 Patricia DREVET, Gilles PETEL, Boris FUMANAL, Aurélie GOUSSET-DUPONT, Hervé
668 COCHARD, Eric BADEL, Jean-Louis JULIEN, Philippe LABEL, and Daniel AUGUIN
669 made a critical examination of the manuscript;

670 Jean-Stéphane VENISSE led the program, co-designed the experiments, obtained the
671 funding, and coordinated and compiled the authors' contributions to the final version of
672 the article. He wrote the final draft of the article and edited it.

673

674 All the authors participated in the analysis of data, and collectively approved all the
675 interpretations of results and related hypotheses.

676

677 **Captions**

678

679 **Figure 1.** Daily variations in the experimental system weight (pot and plant) (black line; kg;
680 analytical balance) and stem diameter (gray line; μm ; LVDT) of *Populus deltoides* in different
681 hydric conditions: well-watered (dark blue), drought treatment (orange, monitored over 8
682 days), and rewatered (light blue, monitored over 10 days). Curves are representative of one
683 tree. Detail in the typical time courses of diurnal variations of experimental system weight
684 and stem diameter in Supplemental Figure S1.

685

686 **Figure 2.** Water relation parameters [(**A**), Ψ_{leaf} , leaf water potential ; (**B**), RWC, relative water
687 content ; (**C**), leaf hydraulic conductance, K_{leaf} ; (**D**), leaf capacitance, C_{leaf}], and gas
688 exchange parameters [(**E**), transpiration flux, E ; (**F**), stomatal conductance, g_s] in well-
689 irrigated plants (dark blue curves), drought treatment (orange curves) and rewatering (light
690 blue curves). Ecophysiological traits were recorded at predawn (5:00 am) and midday (14:00
691 pm). na: g_s non-available data. Data correspond to the mean of seven independent biological
692 experiments. Bars represent the biological standard error. Letters on vertical bars indicate
693 significant differences between treatments [Tukey *post hoc* test after one-way analysis of
694 variance (ANOVA), $p < 0.05$].

695

696 **Figure 3.** Hierarchical clustering of all PIP and TIP transcribed in *Populus deltoides* and
697 transcriptional accumulation patterns of a selected set of *PdPIPs* and *PdTIPs* in leaves of
698 plants challenged by a drying-rewatering cycle. All expressed PIPs and TIPs are shown in
699 Supplemental Figures S3 and S4, respectively. Molecular analyses were performed at
700 predawn (5:00) and midday (14:00). Transcript levels for each gene were estimated using
701 real-time qRT-PCR analyses and normalized by the expression of five housekeeping genes.
702 Relative transcript abundance rates were obtained by the $2^{-\Delta\Delta C_T}$ method with transcript
703 abundances. Data correspond to means of three independent biological replicates. Bars
704 represent the biological standard error. Data not sharing the same letters are significantly
705 different [Tukey *post hoc* test after one-way analysis of variance (ANOVA), $p < 0.05$]. Genes
706 were clustered by a hierarchical clustering algorithm (green, up-expression; red, down-
707 expression, compared with untreated samples). Hierarchical clustering was performed by
708 similarity on \log_2 signal intensity data, and calculated according to Euclidean distance with
709 Ward contrast between expression levels of all PIP and TIP genes (R). Well-watered plants,
710 dark blue; droughted plants, orange; rewatered plants, light blue.

711

712 **Figure 4.** Principal component analysis based on the ecophysiological data (g_s , Ψ_{leaf} , C_{leaf} , E ,
713 K_{leaf} and RWC) and molecular data (PIP and TIP qPCR expression levels). PCA was

714 performed using FactoMineR_1.39, missMDA_1.11 and Factoextra libraries (*R* software;
715 version 3.4.3; *R*-core Team, 2017). PSI, ψ_{leaf} for leaf water potential ; RWC, relative water
716 content ; C_{leaf} , leaf capacitance ; g_s , leaf stomatal conductance ; E , transpiration flux ; K_{leaf} ,
717 leaf conductance ; PIP, plasma intrinsic protein ; TIP, tonoplast intrinsic protein. TIP and PIP
718 nomenclatures used here are contractions in the first and second numbers preceded by the
719 AQP subfamily type, each contraction corresponds to a specific isoform (for example, PIP11
720 for *PdPIP1;1*, etc).

721

722 **Figure 5.** *In situ* localization of selected *PdPIP* and *PdTIP* in midrib (**A, D, G, J, M**), lamina
723 (**B, E, H, K, N**) and BSCs (**C, F, I, L, N** ; extracted from **B, E, H, K, N**) of the leaves from
724 *Populus deltoides*. Pictures correspond to paraffin-embedded transversal sections of leaf
725 samples hybridized with specific antisense probes (i.e. positive, +). Paraffin-embedded
726 transversal sections of leaf samples hybridized with specific sense probes (i.e. negative
727 controls, -) are presented in Supplemental Figure S5. Positive hybridization signals are
728 visualized by violet staining using a DIG-labeled RNA immunodetection system. Arrows
729 indicate differential hybridizations (+ vs -). co, collenchyma ; pa, parenchyma ; co-pa,
730 collenchyma and parenchyma (structures not distinguishable individually) ; ca-ph, cambium
731 and phloem (structures not distinguishable individually) ; sc, sclerenchyma ; pal, palissadic
732 parenchyma of lamina ; sp, spongy parenchyma of lamina ; mv, minor vein used to describe
733 BSC (**C, F, I, L, N**) ; BSC, bundle sheath cells ; xy-ph, xylem and phloem (structures not
734 distinguishable individually). Leaf anatomical features are detailed in Supplemental Figure
735 S6. Bar indicates 100 μ m.

736

737 **Figure 6.** *In vivo* visualization by X-ray microtomography of tissue embolism in a leaf from
738 *Populus deltoides*. Reconstructed cross sections showing embolized (dark points) midrib and
739 vein conduits, and lamina at leaf water potential around (**A**) -0.05 MPa (control sample), (**B**)
740 < -2.5 MPa (drought sample). These water potentials correspond to the mean of ψ_{leaf} from
741 leaves adjacent to those sampled at 5:00 am for X-ray microtomography analysis. Bar
742 indicates 100 μ m. Leaf anatomical features are detailed in Supplemental Figure S6. The leaf
743 in (**B**) was especially sampled for quantitative illustration, highlighting potential wilting
744 symptoms induced by drought just before rewatering. (**C**) Leaf hydraulic model of water flow
745 based on the electric circuit analogy depicts the relationships between hydraulic resistances
746 (R) and the hydraulic capacitances (C) that regulate the balance in water diffusion and
747 storage through different leaf areas when leaf water potential (ψ_{leaf}) is below -2,5 MPa
748 corresponding to severe drought. Downward- and upward-pointing red arrows represent
749 down- and up-regulation, respectively. Line size for " R and C hydraulic components"
750 represents the intensity of their involvement in the hydraulic system. Aquaporins are shown

751 in yellow by their tertiary structure, and their size represents their presence assessed by ISH.
752 Marked AQP in the “xylem” box indicate that they are absent in secondary xylem. g_s ,
753 stomatal conductance; pal, palissadic parenchyma; col, collenchyma; bsc, bundle sheath
754 cell; mv, minor vein.

755

756 **Supplemental Figure S1.** Detail in the typical time courses of diurnal variations of
757 experimental system weight (black line) and stem diameter (gray line) of *Populus deltoides*
758 under full irrigation, in correlation with the photosynthetic active radiation (red line) on day 2
759 of treatment. The experimental design shows the time of molecular sampling and
760 ecophysiological recording (at 5:00 and 14:00), and the watering pulse (well-watered plants
761 in dark blue) and the end of irrigation to start of drought treatment (unwatered plants in
762 orange).

763

764 **Supplemental Figure S2. (A)** Changes in the leaf water potential (Ψ_{leaf}) in relation with the
765 leaf capacitance (C_{leaf}). This representation was generated from the Höfler diagram **(B)**,
766 which shows the changes in whole leaf water potential (Ψ_{leaf}), pressure potential (P_{leaf}) and
767 osmotic potential (π_{leaf}) as a function of relative water content (RWC). TLP, turgor loss point.

768

769 **Supplemental Figure S3.** Transcriptional accumulation patterns of all *PdPIPs* in leaves of
770 plants challenged by a drying-rewatering cycle. Transcript levels for each gene were
771 estimated using real-time qRT-PCR analyses and normalized by the expression of five
772 housekeeping genes. Relative transcript abundance rates were obtained by the $2^{-\Delta\Delta C_T}$
773 method with transcript abundances. Data correspond to means of three independent
774 biological replicates. Bars represent the biological standard error. Data not sharing the same
775 letters are significantly different [Tukey *post hoc* test after one-way analysis of variance
776 (ANOVA), $p < 0.05$]. Well-watered plants, dark blue; droughted plants, orange; rewatered
777 plants, light blue.

778

779 **Supplemental Figure S4.** Transcriptional accumulation patterns of all *PdTIPs* in leaves of
780 plants challenged by a drying-rewatering cycle. Transcript levels for each gene were
781 estimated using real-time qRT-PCR analyses and normalized by the expression of five
782 housekeeping genes. Relative transcript abundance rates were obtained by the $2^{-\Delta\Delta C_T}$
783 method with transcript abundances. Data correspond to means of three independent
784 biological replicates. Bars represent the biological standard error. Data not sharing the same
785 letters are significantly different [Tukey *post hoc* test after one-way analysis of variance
786 (ANOVA), $p < 0.05$]. Well-watered plants, dark blue; droughted plants, orange; rewatered
787 plants, light blue.

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Supplemental Figure S5. *In situ* localization of a selected *PdPIP* and *PdTIP* in midrib and lamina of a leaf from *Populus deltoides*. Photographic images with letters correspond to Figure 5. Paraffin-embedded transversal sections of leaf samples hybridized with specific antisense probes (*i.e.* positive, +) or with sense probes as negative controls (-). Positive hybridization signals are visualized by violet staining using a DIG-labeled RNA immunodetection system. Bar indicates 100 μ m. Leaf anatomical features are detailed in Supplemental Figure S6.

Supplemental Figure S6. (A) Midrib cross-section and (B) anatomic detail of a minor vein within the lamina of a leaf from *Populus deltoides* by light microscopy and toluidine blue staining. (A) ue, upper epidermis ; le, lower epidermis ; co, collenchyma ; pa, parenchyma ; xy2, secondary xylem ; xy1, primary xylem (metaxylem and protoxylem not distinguishable) ; ca, cambium ; ph2, secondary phloem (metaphloem and protophloem not distinguishable) ; sc, sclerenchyma ; mv, minor vein. xy2-xy1 transport the xylem sap ; xylem sap-ca-ph2 constitute the vessel tissue (*i.e.* vascular bundle) ; sc-pa-co-le/ue constitute the extravessel tissue ; sap vessel tissue-extravessel tissue constitute the midrib. (B) ue, upper epidermis ; pal, palissadic parenchyma of lamina ; sp, spongy parenchyma of lamina ; bsc, bundle sheath cells (area delimited by the red line) ; xy/ph, xylem/phloem (structures individually not distinguishable).

825 **Supplemental Table S1.** Primers used for qPCR amplification and the MIP transcript tissue
 826 locations using *in situ* hybridization.

	Locus name <i>Populus deltoides</i> WV94 v2.1	Primer sequences	
		Forward	Reverse
Actine 2	Podel.19G009700.1	GAAGTGCTTCTAAGTTCTACAAG	CTCAATAAATTCTCCATATCAACC
EF1α	Podel.08G051800.1	GTCTGTTGAGATGCACCACG	CAATGTGACAGGTGTGGCAG
SAND	Podel.09G012400.1	CATGATAAAGGCAACGGGGCG	CTGTGTTACAAGATATTTTTGGG
TIP41-like	Podel.01G317800.1	CAGTGAAGTGCAAATCCTATTG	CTTACAAGTTACTGTGGACCAC
UP2	Podel.02G140600.1	TATCGTCTTGTGACAATTTTTAG	GGAAGTCCTGGCGTAATGA
PdPIP1;1	Podel.08G080400.1	CAAGAAGTGATGAYTGTGATGC	ACGCTACTGT TATAGCACGC
PdPIP1;2	Podel.03G137600.1	TTCAAGAGCAGAGCTTAATTTTC	TTCAAAAAGCTAGATAAATTACAC
PdPIP1;3	Podel.10G194700.1	AAGAAGTGATATATGATTATGG	GATTTGAAGAAACACGTAATTGCTA
PdPIP2;1	Podel.09G141800.1	TGCTAACATGGTGGCTCCC	GATCATCCCAGGCTTTCTTATC
PdPIP2;2	Podel.04G178700.1	TGGTACACTTGGCCACAATC	GCTAATGCTCCCACAAATGG
PdPIP2;3	Podel.16G093500.1	ATTCAAGTTATGGAACGTACGC	AGCTCACAACCATGACATAAC
PdPIP2;4	Podel.10G227600.1	CCAACGGTTTTAAATCTCGGTTT	ACCGAAAGGGATAATAAAGGG
PdPIP2;5	Podel.06G138200.1	TGTGTACATTATGGTGTCTGTG	CAAAGAAGCAGCACTACCTGAGAC
PdPIP2;6	Podel.08G048200.1	ATCCCAATCACAGGAAGTGG	CTATGGCTGCACCAATGAAG
PdPIP2;7	Podel.09G012100.1	ATGACCATTGGCTCTTCTGG	GGCTTTAAGCATTGCTCCTG
PdPIP2;8	Podel.05G122300.1	GCAATCCTACTAGCTAAGGC	ACCCACATATCAAGTTGAGAC
PdPIP2;9	Podel.05G122200.1	ACTGGAAGTGGCATCAATCC	CCAACCCAGAAAATCCACAG
PdPIP2;10	Podel.06G138300.1	CACAGTCGTGGTCAAGATGTC	ACTAGTTGATTATGAGATAGGGAG
PdTIP1;1	Podel.01G246100.1	TGCTGCCATTGTCTACGAGGTCAT	CACCACCACAGACGTGTCTTTC
PdTIP1;2	Podel.09G026800.1	TGCTGCCATTGTCTACGAGGCTG	AACAACACCAACATCATCCAAACC
PdTIP1;3	Podel.10G214800.1	GATTGGAAGTCCAGCTTTTGGAT	AATTTCCCTTCTTTGGATCCACT
PdTIP1;4	Podel.08G061500.1	ATTGGAACACCAGCTTTTGGGC	AATTTCCCTTCTTCGGATCCACC
PdTIP1;5	Podel.16G102900.1	ATTGTGGGTGCTAACATTCTAGTC	CCACCAACCAGTGGTCCGGC
PdTIP1;6	Podel.06G131300.1	CAACCACTGGGTCTACTGGGCA	GAAAGAAAAAGCCGGGCACTG
PdTIP1;7	Podel.09G003500.1	CGAGTTAATCTTCATCAGCCACAC	GGAAAAAATTCACGTAAGTCTTAAAC
PdTIP1;8	Podel.04G222000.1	AGCTTATCTTCATGAGCCACAGTAC	ACGAATCCAACGTAGTCCAAACG
PdTIP2;1	Podel.01G197500.1	TCTACTGGGCTGGGCCTCTTG	CAAGTGAAGTTATTAGAAGTCTCC
PdTIP2;2	Podel.07G052500.1	TCTACTGGGTTGGGCCTCTTA	ATTTTAGACTGACTTAGAACTCATA
PdTIP2;3	Podel.03G081200.1	TTGCTTCAGGGATGAGTGCTATC	TTCAGAAACTGGGGCAGGGCC
PdTIP2;4	Podel.01G168400.1	TCGCTTCAGGGATGAGTGCTGTT	TTCGGAACCTGGGGCAGGGGT
PdTIP3;1	Podel.18G157700.1	GAGCATTTGGGCCTGCTCTAG	GGTGAGCTACTGGCTCTGCTG
PdTIP3;2	Podel.17G170600.1	GAGCTTTTGGGCCTGCTTTAA	GGCGAGGTAAGTGGCTCTGTGG
PdTIP4;1	Podel.06G131300.1	ATGGGCTAGGTCCAATGCTGAC	GAAGAGGACGATGAGATCTTGTG

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830 **References**

- 831 Aasamaa, K., Sober, A., 2001. Hydraulic conductance and stomatal sensitivity to changes of
832 leaf water status in six deciduous tree species. *Biologia Plantarum* 44, 65-73.
- 833 Aasamaa, K., Sober, A., Rahi, M., 2001. Leaf anatomical characteristics associated with
834 shoot hydraulic conductance, stomatal conductance and stomatal sensitivity to changes of
835 leaf water status in temperate deciduous trees. *Australian Journal of Plant Physiology* 28,
836 765-774.
- 837 Anderberg, H.I., Kjellbom, P., Johanson, U., 2012. Annotation of *Selaginella moellendorffii*
838 major intrinsic proteins and the evolution of the protein family in terrestrial plants. *Frontiers in*
839 *Plant Science* 3: 33.
- 840 Attia Z., Domec, J.C., Oren, R., Way, D.A., Moshelion, M., 2015. Growth and physiological
841 responses of isohydric and anisohydric poplars to drought. *J. Exp. Bot.* 66, 4373-4381.
- 842 Bartlett, M.K., Scoffoni, C., Sack, L., 2012. The determinants of leaf turgor loss point and
843 prediction of drought tolerance of species and biomes: a global meta-analysis. *Ecology Letter*
844 15, 393-405.
- 845 Bartlett, M.K., Klein, T., Jansen, S., Choat, B., Sack, L., 2016. The correlations and
846 sequence of plant stomatal, hydraulic, and wilting responses to drought. *Proc Natl Acad Sci*
847 *USA* 113, 13098-13103.
- 848 Begg, J.E., Turner, N.C., 1970. Water potential gradients in field tobacco. *Plant Physiology*
849 46, 343-346.
- 850 Ben Baaziz, K., Lopez, D., Rabot, A., Combes, D., Gousset, A., Bouzid, S. et al., 2012. Light-
851 mediated K_{leaf} induction and contribution of both the PIP1s and PIP2s aquaporins in five tree
852 species: walnut (*Juglans regia*) case study. *Tree Physiol.* 32, 423-434.
- 853 Blackman, C.J., Brodribb, T.J., Jordan, G.J., 2009. Leaf hydraulics and drought stress:
854 response, recovery and survivorship in four woody temperate plant species. *Plant, Cell and*
855 *Environment.* 32, 1584-1595.
- 856 Brodribb, T.J., Skelton, R.P., McAdam, S.A.M., Bienaimé, D., Lucani, C.J., Marmottant, P.,
857 2016a. Visual quantification of embolism reveals leaf vulnerability to hydraulic failure. *New*
858 *Phytologist* 209, 1403-1409.
- 859 Brodribb, T.J., Bienaimé D., Marmottant, P., 2016b. Revealing catastrophic failure of leaf
860 networks under stress. *PNAS* 113, 4865-4869.
- 861 Brunel, N., Leduc, N., Poupard, P., Simoneau, P., Mauget, J.C., Viemont, J.D., 2002.
862 KNAP2, a class I KN1-like gene is a negative marker of bud growth potential in apple trees

863 (*Malus domestica* [L.] Borkh.). Journal of Experimental Botany 53, 2143-2149.

864

865 Buckley, T.N., John, G.P., Scoffoni, C., Sack, L., 2015. How does leaf anatomy influence
866 water transport outside the xylem? Plant Physiology 168, 1616-1635.

867 Bunce, J.A., 2006. How do leaf hydraulics limit stomatal conductance at high water vapour
868 pressure deficits? Plant Cell Environ 29, 1644-1650.

869 Caringella, M.A., Bongers, F.J. Sack, L., 2015. Leaf hydraulic conductance varies with vein
870 anatomy across *Arabidopsis thaliana* wild-type and leaf vein mutants. Plant, Cell &
871 Environment 38, 2735-2746.

872 Chang, S.J., Puryear, J., Cairney, J., 1993. A simple and efficient method for isolating RNA
873 from pine trees. Plant Mol. Biol. Rep. 11, 113-116.

874 Chaumont, F., Tyerman, S.D., 2014. Aquaporins: highly regulated channels controlling plant
875 water relations. Plant Physiology 164, 1600-1618.

876 Choat, B., Jansen, S., Brodribb, T.J., et al, 2012. Global convergence in the vulnerability of
877 forests to drought. Nature 491, 752-755

878 Czechowski, T., Stitt, M., Altmann, T., Udvardi, M.K. and Scheible, W.R., 2005 Genome-wide
879 identification and testing of superior reference genes for transcript normalization in
880 *Arabidopsis*. Plant Physiol. 139, 5-17.

881 Danielson, J.A.H., Johanson, U., 2008. Unexpected complexity of the aquaporin gene family
882 in the moss *Physcomitrella patens*. BMC Plant Biology 8, 45-60.

883 De Schepper, V., van Dusschoten, D., Copini, P., Jahnke, S., Steppe, K. 2012. MRI links
884 stem water content to stem diameter variations in transpiring trees. Journal of Experimental
885 Botany 63, 2645-2653.

886 Dobra, J., Motyka, V., Dobrev, P., Malbeck, J., Prasil, I.T., Haisel, D., Gaudinova, A.,
887 Havlova, M., Gubis, J., Vankova, R., 2010. Comparison of hormonal responses to heat,
888 drought and combined stress in tobacco plants with elevated proline contents. Journal of
889 Plant Physiology 167, 1360-1370.

890 Fetter, K., Van Wilder, V., Moshelion, M., Chaumont, F., 2004. Interactions between plasma
891 membrane aquaporins modulate their water channel activity. Plant Cell 16, 215-228.

892 Ferrio, J.P., Pou, A., Florez-Sarasa, I., Gessler, A., Kodama, N., Flexas, J., Ribas-Carbo, M.,
893 2012. The Peclet effect on leaf water enrichment correlates with leaf hydraulic conductance
894 and mesophyll conductance for CO₂. Plant Cell and Environment 35, 611-625.

895 Fichot, R., Brignolas, F., Cochard, H., Ceulemans, R., 2015. Vulnerability to drought-induced
896 cavitation in poplars: synthesis and future opportunities. *Plant Cell Environ* 38, 1233-1251.

897 Garavillon-Tourmayre, M., Gousset-Dupont, A., Gautier, F., Benoit, P., Conchon, P., Souchal,
898 R., Lopez, D., Petel, G., Venisse, J.S., Bastien, C., Label, P., Fumanal, B., 2017. Integrated
899 drought responses of black poplar: how important is phenotypic plasticity?, *Physiologia*
900 *Plantarum*, 163, 30-44.

901 Galmés, J., Pou, A., Alsina, M.M., Tomas, M., Medrano, H., Flexas, J., 2007. Aquaporin
902 expression in response to different water stress intensities and recovery in Richter-110 (*Vitis*
903 sp.): relationship with ecophysiological status. *Planta* 226, 671-681.

904 Gattolin, S., Sorieul, M., Frigerio, L., 2011. Mapping of tonoplast intrinsic proteins in maturing
905 and germinating *Arabidopsis* seeds reveals dual localization of embryonic TIPs to the
906 tonoplast and plasma membrane. *Molecular Plant*. 4, 180-189.

907 Griffiths, H., Weller, G., Toy, L.F.M., Dennis, R.J., 2013. You're so vein: Bundle Sheath
908 physiology, phylogeny and evolution in C3 and C4 plants. *Plant, Cell & Environment* 36, 249-
909 261.

910 Gupta, A.B., Sankararamkrishnan R., 2009. Genome-wide analysis of major intrinsic
911 proteins in the tree plant *Populus trichocarpa*: characterization of XIP subfamily of
912 aquaporins from evolutionary perspective. *BMC Plant Biology* 20, 134.

913 Heckwolf, M., Pater, D., Hanson, D.T., Kaldenhoff, R., 2011. The *Arabidopsis thaliana*
914 aquaporin AtPIP1;2 is a physiologically relevant CO₂ transport facilitator. *The Plant Journal*
915 67, 795-804.

916 Heinen, R.B., Ye, Q., Chaumont, F., 2009. Role of aquaporins in leaf physiology. *Journal of*
917 *Experimental Botany* 60, 2971-2985.

918 Heymann, J.B., Engel, A., 1999. Aquaporins: phylogeny, structure, and physiology of water
919 channels. *News Physiol Sci* 14, 187-193.

920 Hochberg, U., Bonel, A.G., David-Schwartz, R., Degu, A., Fait, A., Cochard, H., Peterlunger,
921 E., Herrera, J.C., 2017. Grapevine acclimation to water deficit: the adjustment of stomatal
922 and hydraulic conductance differs from petiole embolism vulnerability. *Planta* 245, 1091-
923 1104.

924 Hochberg, U., Windt, C.W., Ponomarenko, A., Zhang, Y.J., Gersony, J., Rockwell, E.F.,
925 Holbrook, N.M., 2017. Stomatal Closure, Basal Leaf Embolism, and Shedding Protect the
926 Hydraulic Integrity of Grape Stems. *Plant Physiology* 174, 764-775.

927 Jackson, M.B., Saker, L.R., Crisp, C.M., Else, M.A., Janowiak, F., 2003. Ionic and pH

928 signalling from roots to shoots of flooded tomato plants in relation to stomatal closure. *Plant*
929 *and Soil* 253, 103-113.

930 Javelle, M., Vernoud, V., Rogowsky, P.M., Ingram, G.C., 2011. Epidermis: the formation and
931 functions of a fundamental plant tissue. *New Phytol.* 189, 17-39.

932 Josse, J., Husson, F., 2016. missMDA: A Package for Handling Missing Values in
933 Multivariate Data. Analysis. *Journal of Statistical Software*, 70, 1-31.

934 Kassambara, A., Mundt, F., 2017. factoextra: Extract and Visualize the Results of
935 Multivariate Data Analyses. R package version 1.0.5. [https://CRAN.R-](https://CRAN.R-project.org/package=factoextra)
936 [project.org/package=factoextra](https://CRAN.R-project.org/package=factoextra)

937 Khan, S.H., Ahmad, N., Ahmad, F., Kumar, R., 2010. Naturally Occurring Organic
938 Osmolytes: From Cell Physiology to Disease Prevention *IUBMB Life* 62, 891-895.

939 Koide, R.T., Robichaux, R.H., Morse, S.R., Smith, C.M., 1989. Plant water status, hydraulic
940 resistance and capacitance. In *Plant Physiological Ecology: Field Methods and*
941 *Instrumentation* (eds R.W. Pearcy, J.R. Ehleringer, H.A. Mooney & P.W. Rundel), pp. 161-
942 183. Chapman and Hall, New York, NY, USA.

943 Larchevêque, M., Maurel, M., Desrochers A., Larocque, G.R., 2011. How does drought
944 tolerance compare between two improved hybrids of balsam poplar and an unimproved
945 native species? *Tree Physiology* 31, 240-249.

946 Laur, J., Hacke, U.G., 2014. Exploring *Picea glauca* aquaporins in the context of needle
947 water uptake and xylem refilling. *New Phytologist* 203, 388-400.

948 Le, S., Josse, J., Husson F., 2008. FactoMineR: An R Package for Multivariate Analysis.
949 *Journal of Statistical Software* 25, 1-18.

950 Lee, S.J., Murphy, C.T., Kenyon, C., 2009. Glucose shortens the life span of *C. elegans* by
951 downregulating DAF-16/FOXO activity and aquaporin gene expression. *Cell Metab.* 10, 379-
952 391.

953 Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time
954 quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 25, 402-408.

955 Liu, X., Bandyopadhyay, B., Nakamoto, T., Singh, B., Liedtke, W., Melvin, J.E., Ambudkar, I.
956 2006. A role for AQP5 in activation of TRPV4 by hypotonicity: concerted involvement of
957 AQP5 and TRPV4 in regulation of cell volume. *J Biol Chem.* 281, 15485-15495.

958 Lopez, D., Bronner, G., Brunel, N., Auguin, D., Bourgerie, S., Brignolas, F., Carpin, S.,
959 Tournaire-Roux, C., Maurel, C., Fumanal, B., Martin, F., Sakr, S., Label, P., Julien, J.L.,
960 Gousset-Dupont, A., Venisse, J.S., 2012. Insights into *Populus* XIP aquaporins: evolutionary

961 expansion, protein functionality, and environmental regulation. *Journal of Experimental*
962 *Botany* 63, 2217-2230.

963 Lopez, D., Venisse, J.S., Fumanal, B., Chaumont, F., Guillot, E., Daniels, M.J., Cochard, H.,
964 Julien, J.L., Gousset-Dupont, A., 2013. Aquaporins and Leaf Hydraulics: Poplar Sheds New
965 Light. *Plant Cell Physiology* 54, 1963-1975.

966 Lopez, D., Amira, M.B., Brown, D., Muries, B., Brunel-Michac, N., Bourgerie, S., Porcheron,
967 B., Lemoine, R., Chrestin, H., Mollison, E., Di Cola, A., Frigerio, L., Julien, J.L., Gousset-
968 Dupont, A., Fumanal, B., Label, P., Pujade-Renaud, V., Auguin, D., Venisse J.S., 2016. The
969 *Hevea brasiliensis* XIP aquaporin subfamily: genomic, structural and functional
970 characterizations with relevance to intensive latex harvesting. *Plant Molecular Biology* 91,
971 375-396.

972 Martin, C.E., Lin, T.C., Lin, K.C., Hsu, C.C., Chiou, W.L., 2004. Causes and consequences of
973 high osmotic potentials in epiphytic higher plants. *Journal of Plant Physiology* 161, 1119-
974 1124.

975 Martin-StPaul, N., Delzon, S., Cochard, H., 2017. Plant resistance to drought depends on
976 timely stomatal closure. *Ecology Letters*, doi: 10.1111/ele.12851.

977 Mertz, R.A., Brutnell, T.P., 2014. Bundle sheath suberization in grass leaves: multiple
978 barriers to characterization. *Journal of Experimental Botany* 65, 3371-3380.

979 Maurel, C., Tacnet, F., Guclu, J., Guern, J., Ripoche, P., 1997. Purified vesicles of tobacco
980 cell vacuolar and plasma membrane exhibit dramatically different water permeability and
981 water channel activity. *PNAS* 94, 7103-7108.

982 Nobel, P.S., 2006. Parenchyma-chlorenchyma water movement during drought for the
983 hemiepiphytic cactus *Hylocereus undatus*. *Annals of Botany* 97, 469-474.

984 Ohtsuka, A., Sack, L., Taneda, H., 2017. Bundle sheath lignification mediates the linkage of
985 leaf hydraulics and venation. *Plant Cell Environ.* 18, 1-12.

986 Pfaffl, M.W., Tichopad, A., Prgomet, C., Neuvians, T.P., 2004. Determination of stable
987 housekeeping genes, differentially regulated target genes and sample integrity:
988 BestKeeper–Excel-based tool using pair-wise correlations. *Biotechnology Letter* 26, 509-515.

989 Pou, A., Medrano, H., Flexas, J., Tyerman, S.D., 2013. A putative role for TIP and PIP
990 aquaporins in dynamics of leaf hydraulic and stomatal conductances in grapevine under
991 water stress and re-watering. *Plant Cell Environment* 36, 828-843.

992 Prado, K., Maurel, C., 2013. Regulation of leaf hydraulics: from molecular to whole plant
993 levels. *Frontiers in Plant Science* 4, 58-60.

994 Prado, K., Boursiac, Y., Tournaire-Roux, C., Monneuse, J.M., Postaire, O., Da Ines, O.,
995 Maurel, C., 2013. Regulation of *Arabidopsis* leaf hydraulics involves light-dependent
996 phosphorylation of aquaporins in veins. *Plant Cell* 25, 1029-1039.

997 R Core Team (2017). R: A language and environment for statistical computing. R Foundation
998 for Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/>.

999 Rockwell, F.E., Holbrook, N.M., 2017. Leaf Hydraulic Architecture and Stomatal
1000 Conductance: A Functional Perspective. *Plant Physiology* 174, 1996-2007.

1001 Sack, L., Melcher, P.J., Zwieniecki, M.A., Holbrook, N.M., 2002. The hydraulic conductance
1002 of the angiosperm leaf lamina: a comparison of three measurement methods. *Journal of*
1003 *Experimental Botany* 53, 2177-2184.

1004 Sack, L., Holbrook, N.M., 2006. Leaf hydraulics. *Annu. Rev. Plant Biol.* 57, 361-381.

1005 Sack, L., Cowan, P.D., Jaikumar, N., Holbrook, N.M., 2003. The 'hydrology' of leaves: co-
1006 ordination of structure and function in temperate woody species. *Plant Cell and Environment*
1007 26, 1343-1356.

1008 Sack, L., Pasquet-Kok, J., 2011. [http://www.publish.csiro.au/prometheuswiki/tiki-](http://www.publish.csiro.au/prometheuswiki/tiki-pagehistory.php?page=Leaf%20pressure-volume%20curve%20parameters&preview=16)
1009 [pagehistory.php?page=Leaf pressure-volume curve parameters&preview=16](http://www.publish.csiro.au/prometheuswiki/tiki-pagehistory.php?page=Leaf%20pressure-volume%20curve%20parameters&preview=16).

1010 Sack, L., Scoffoni, C., 2013. Leaf venation: structure, function, development, evolution,
1011 ecology and applications in the past, present and future. *New Phytologist* 198, 983-1000.

1012 Sade, N., Shatil-Cohen, A., Attia, Z., Maurel, C., Boursiac, Y., Kelly, G., Granot, D., Yaaran,
1013 A., Lerner, S., Moshelion, M., 2014. The role of plasma membrane aquaporins in regulating
1014 the bundle sheath-mesophyll continuum and leaf hydraulics. *Plant Physiology* 166, 1609-
1015 1620.

1016 Sade, N., Moshelion, M. 2016. Plant aquaporins and abiotic stress. In *Plant Aquaporins:*
1017 *From Transport to Signalling*; Chaumont, F., Tyerman, S., Eds.; Springer-Verlag: Berlin-
1018 Heidelberg, Germany.

1019 Sakr, S., Alves, G., Morillon, R., Maurel, K., Decourteix, M., Guillot, A., Fleurat-Lessard, P.,
1020 Julien, J.L., Chrispeels, M.J., 2003. Plasma membrane aquaporins are involved in winter
1021 embolism recovery in walnut tree. *Plant Physiology* 133, 630-641.

1022 Schmidt, J.E., Kaiser, W.M., 1987. Response of the succulent leaves of *Peperomia*
1023 *magnoliaefolia* to dehydration. *Plant Physiology* 83, 190-194.

1024 Scholz, F.G., Bucci, S.J., Hoffmann, W.A., Meinzer, F.C., Goldstein, G., 2010. Hydraulic lift in
1025 a neotropical savanna: experimental manipulation and model simulations. *Agric For Meteorol*
1026 150, 629-639

- 1027 Scoffoni, C., Jansen, S., 2016. I Can See Clearly Now - Embolism in Leaves. Trends in Plant
1028 Science 21, 723-725.
- 1029 Scoffoni, C., Albuquerque, C., Brodersen, C.R., Townes, S.V., John, G.P., Cochard, H.,
1030 Buckley, T.N., McElrone, A.J., Sack, L., 2017a. Leaf vein xylem conduit diameter influences
1031 susceptibility to embolism and hydraulic decline. New Phytologist 213, 1076-1092.
- 1032 Scoffoni, C., Albuquerque, C., Brodersen, C.R., Townes, S.V., Grace, P.J., Bartlett, M.K.
1033 Buckley, T.N., McElrone, A.J., Sack, L., 2017b. Outside-Xylem Vulnerability, Not Xylem
1034 Embolism, Controls Leaf Hydraulic Decline during Dehydration. Plant Physiol, 173,1997-
1035 1210.
- 1036 Secchi, F., Maciver, B., Zeidel, M.L., Zwieniecki, M.A., 2009. Functional analysis of putative
1037 genes encoding the PIP2 water channel subfamily *Populus trichocarpa*. Tree Physiology 29,
1038 1467-1477.
- 1039 Secchi, F., Zwieniecki, M.A., 2010. Patterns of PIP gene expression in *Populus trichocarpa*
1040 during recovery from xylem embolism suggest a major role for the PIP1 aquaporin subfamily
1041 as moderators of refilling process. Plant Cell Environ 33, 1285-1297.
- 1042 Shatil-Cohen, A., Attia, Z. Moshelion, M., 2011. Bundle-sheath cell regulation of xylem-
1043 mesophyll water transport via aquaporins under drought stress: a target of xylem-borne
1044 ABA? Plant J 67, 72-80.
- 1045 Smith, J.A.C., Schulte, P.J., Nobel, P.S., 1987. Water flow and water storage in *Agave*
1046 *deserti*: osmotic implications of Crassulacean acid metabolism. Plant Cell and Environment
1047 10, 639-648.
- 1048 Sommerville, K.E., Sack, L., Ball, M.C., 2012. Hydraulic conductance of *Acacia phyllodes*
1049 (foliage) is driven by primary nerve (vein) conductance and density. Plant, Cell and
1050 Environment 35, 158-168.
- 1051 Steppe, K., De Pauw, D.J.W., Lemeur, R., Vanrolleghem, P.A. 2006. A mathematical model
1052 linking tree sap flow dynamics to daily stem diameter fluctuations and radial stem growth.
1053 Tree Physiology 26, 257-273.
- 1054 Terashima, I., 1992. Anatomy of non-uniform leaf photosynthesis. Photosynth Res. 31, 195-
1055 212.
- 1056 Tissue, D.T., Yakir, D., Nobel, P.S., 1991. Diel water movement between parenchyma and
1057 chlorenchyma of two desert CAM plants under dry and wet conditions. Plant Cell and
1058 Environment 14, 407-413.

1059 Tyree, M.T., Hammel, H.T. 1972. The measurement of the turgor pressure and the water
1060 relations of plants by the pressure-bomb technique. J. Exp. Bot. 23, 267-282.

1061 Tyree, M.T., Alexander, J., Machado, J.L., 1992. Loss of hydraulic conductivity due to water
1062 stress in intact juveniles of *Quercus rubra* and *Populus deltoides*. Tree Physiology 10, 411-
1063 415.

1064 van den Honert, T.H., 1948. Water transport in plants as a catenary process. Discuss
1065 Faraday Soc 3, 146-153.

1066 Vendramini, F., Diaz, S., Gurvich, D.E., Wilson, P.J., Thompson, K., Hodgson, J.G., 2002.
1067 Leaf traits as indicators of resource-use strategy in floras with succulent species. New
1068 Phytologist 154, 147-157.

1069 Vitali, M., Cochard, H., Gambino, G., Ponomarenko, A., Perrone, I., Lovisolo, C., 2016.
1070 VvPIP2;4N aquaporin involvement in controlling leaf hydraulic capacitance and resistance in
1071 grapevine. Physiologia Plantarum. 158, 284-296.

1072 Warnes, G.R., Ben Bolker, W., Bonebakker, L., Gentleman, R., Liaw, W.H.A., Lumley, T.,
1073 Maechler, M., Magnusson, A., Moeller, S., Schwartz, M., Venables, B., 2016. gplots: Various
1074 R Programming Tools for Plotting Data. R package version 3.0.1.

1075 Wheeler, J.K., Huggett, B.A., Tofte, A.N., Rockwell, F.E., Holbrook, N.M., 2013. Cutting
1076 xylem under tension or supersaturated with gas can generate PLC and the appearance of
1077 rapid recovery from embolism. Plant, Cell and Environment 36, 1938-1949.

1078 Williams M.L., Thomas B.J., Farrar J.F., Pollock C.J., 1993. Visualizing the distribution of
1079 elements within Barley leaves by energy-dispersive x-ray image maps (Edx Maps). New
1080 Phytologist 125, 367-372.

1081 Xu, M., Zhang, B., Su, X., Zhang, S., Huang, M., 2011. Reference gene selection for
1082 quantitative real-time polymerase chain reaction in Populus. Anal. Biochem. 408, 337-339.

1083 Zelazny, E., Borst, J.W., Muylaert, M., Batoko, H., Hemminga, M.A., Chaumont, F., 2007.
1084 FRET imaging in living maize cells reveals that plasma membrane aquaporins interact to
1085 regulate their subcellular localization. Proc Natl Acad Sci USA 104, 12359-12364.