

Aquaporins and water control in drought-stressed poplar leaves: A glimpse into the extraxylem vascular territories

Beatriz Muries Bosch, Robin Mom, Pierrick Benoit, Nicole Brunel-Michac, Hervé Cochard, Patricia Roeckel-Drevet, Gilles Petel, Eric Badel, Boris Fumanal, Aurelie Gousset, et al.

▶ 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

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers. L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

MURIES Beatriz^a, MOM Robin^a, BENOIT Pierrick^a, BRUNEL-MICHAC Nicole^a, COCHARD
 Hervé^a, DREVET Patricia^a, PETEL Gilles^a, BADEL Eric^a, FUMANAL Boris^a, GOUSSET DUPONT Aurélie^a, JULIEN Jean-Louis^a, LABEL Philippe^a, AUGUIN Daniel^b, VENISSE Jean Stéphane^a*
 Aquaporins and water control in drought-stressed poplar leaves: a

glimpse into the extraxylem vascular territories

- 7
- 8

20 AUTHORS' E-MAIL ADDRESSES:

- 21 bmuries@gmail.com
- 22 robin.mom@etu.uca.fr
- 23 pierrickfrom@free.fr
- 24 nicole.brunel@uca.fr
- 25 herve.cochard@inra.fr
- 26 patricia.drevet@uca.fr
- 27 gilles.petel@uca.fr
- 28 eric.badel@inra.fr
- 29 boris.fumanal@uca.fr
- 30 aurelie.gousset@uca.fr
- 31 Jean-Louis.JULIEN@uca.fr
- 32 philippe.label@uca.fr
- 33 auguin@univ-orleans.fr
- 34 j-stephane.venisse@uca.fr

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 aguaporins. 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





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.
- Specific sub-tissue water allocation correlates with differential PIP and TIP aquaporin
 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 ($\Psi_{\rm w}$). 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, $K_{\rm h}$) 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 "chlorenchyma"), displaying contrasting capacitance between these two distinct but 134 neighboring cell types: high C for water storage parenchyma cells and lower C for 135 chlorenchyma (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 μ mol m⁻² 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 µmol m⁻² 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 ($\Psi_{\text{leaf}} < -3,5$ 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 Ψ_{leaf} = 0.0 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 μ m) 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- BDM-15, readability from 0.1 g, PCE, France) connected to a data acquisition system
(CR1000, Campbell Scientific). For the ecophysiological and molecular analysis, fully
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 Scholander pressure chamber (PMS Instrument Company, Model 1505 D, Albany, OR, 226 227 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 (κ_{leaf}) was 230 assessed according to Attia et al. (2015), and calculated by using the equation $K_{\text{leaf}} = E I$ 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 <mark>area (m²) was measured using an ImageJ macro after scanning.</mark> 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) x 100] were monitored as the tissue dehydrated (where FW, DW, and TW are the fresh, dry and turgid masses, 245 246 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 unit leaf area and unit water content (C_{leaf} ; $\Delta \theta / \Delta \Psi . s^{-1}$) was obtained from these PV curves 249 250 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)

One leaf from three trees (which corresponds to three independent biological replicates) was
 sampled at 5:00am and 2:00pm, at Days -1, 6, 7, 8, 9, 10, 11 and 18 of the experimentation.
 Sampled leaves were depetiolated and immediately frozen in liquid nitrogen for subsequent
 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 System for RT-PCR (InvitrogenTM) and with oligo-dT, following the manufacturer's 271 272 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 $C_{\rm t}$ method. PCR was 275 performed in a 96-well plate with a total volume of 15 µl, containing 10 µl of MESA GREEN 276 gPCR 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, EF1a, 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 selected from a number of candidates using the software application BestKeeper (Pfaffl et al., 2004), first to determine the most suitable reference genes from nine widely used housekeeping genes (Czechowski et al., 2005; Xu et al., 2011), and then to estimate a BestKeeper Index used as a calibrator. Lastly, signal intensity data for all transcribed PIP and TIP genes were hierarchically clustered (Ward contrast, Euclidean distance), with R (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 *p*GEM[®] 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) containing silicone oil. The field of view was $\sim 2.5 \times 2.5 \times 2.5 \text{ mm}^3$ and covered the full cross 324 325 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 resolution of 2.5 \times 2.5 \times 2.5 μ m³ per voxel, were reconstructed by Datos x 2.0 (Phoenix, 328

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, using the Scholander pressure chamber described above. The water potentials indicated on 335 336 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 ± 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 molecular data were aggregated. Ecophysiological variables were Ψ_{leaf} , RWC, K_{leaf} , C_{leaf} , E345 346 and gs. 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 (Day 8). These values matched a π_{tto} value at -1.67 MPa and a RWC_{TLP} of 86% 361 362 (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 Ψ_{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 CO₂, 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 guestioned 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 homeohydry 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 survive under drought (Javelle et al., 2011). Stem diameters only broke down substantially 418 419 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 of423 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.

It is commonly accepted that aquaporins allow transmembrane water movement along osmotic or hydraulic gradients. The fact that a set of AQP is up-regulated during water deprivation suggests that leaves keep a constant fine-tuned balance between water loss and water uptake. However, because drought impedes water uptake, we speculate that a controlled adjustment of the whole AQP pool may occur differentially in various cell/leaf subcompartments and limit a catastrophic process of tissue death. It is thus of interest to know how each AQP is involved in adjusting dynamic leaf hydraulic trade-off between main useand 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- Ψ_{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?

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

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 quantitative evaluation of this phenomenon remains uncertain on excision of small organs that are under strong hydraulic tensions and there is always a risk of overestimating the xylem embolism in the samples (Wheeler et al., 2013). Similarly, we cannot monitor the hydration level of the different leaf tissues. Lastly, the water potentials displayed on the figure 6AB are only indicative and correspond to the mean of the Ψ_{leaf} from the leaves adjacent to 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 ($\Psi_{\text{leaf}} < -2.5 \text{ MPa}$) 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, 500 general metabolic performance depresses and there is an considerable emergence of 501 "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.

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

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

- 658
- 659
- 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;
- Patricia DREVET, Gilles PETEL, Boris FUMANAL, Aurélie GOUSSET-DUPONT, Hervé
 COCHARD, Eric BADEL, Jean-Louis JULIEN, Philippe LABEL, and Daniel AUGUIN
 made a critical examination of the manuscript;
- Jean-Stéphane VENISSE led the program, co-designed the experiments, obtained the
 funding, and coordinated and compiled the authors' contributions to the final version of
 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

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

685

Figure 2. Water relation parameters [(A), Ψ_{leaf} , leaf water potential ; (B), RWC, relative water 686 687 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 real-time gRT-PCR analyses and normalized by the expression of five housekeeping genes. 701 Relative transcript abundance rates were obtained by the $2^{-\Delta\Delta C_T}$ method with transcript 702 703 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 log₂ 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

Figure 4. Principal component analysis based on the ecophysiological data (gs, Ψ_{leaf} , C_{leaf} , E, 713 K_{leaf} and RWC) and molecular data (PIP and TIP *q*PCR expression levels). PCA was performed using FactoMineR_1.39, missMDA_1.11 and Factoextra libraries (*R* software; version 3.4.3; *R*-core Team, 2017). PSI, Ψ_{leaf} for leaf water potential ; RWC, relative water content ; C_{leaf} , leaf capacitance ; g_s , leaf stomatal conductance ; *E*, transpiration flux ; K_{leaf} , leaf conductance ; PIP, plasma intrinsic protein ; TIP, tonoplast intrinsic protein. TIP and PIP nomenclatures used here are contractions in the first and second numbers preceded by the AQP subfamily type, each contraction corresponds to a specific isoform (for example, PIP11 for *Pd*PIP1;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 µm.

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 µm. 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. Aguaporins are shown

in yellow by their tertiary structure, and their size represents their presence assessed by ISH. Marked AQP in the "xylem" box indicate that they are absent in secondary xylem. g_{s} , stomatal conductance; pal, palissadic parenchyma; col, collenchyma; bsc, bundle sheath cell; mv, minor vein.

755

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

763

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

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.

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 µm. 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).

Supplemental Table S1. Primers used for qPCR amplification and the MIP transcript tissue

826 locations using *in situ* hybridization.

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

	Locus name	Primer sequences	
	Populus deltoides WV94 v2.1	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	CAGTGAAAGTGCAAATCCTATTG	CTTACAAGTTACTGTGGACCAC
UP2	Podel.02G140600.1	TATCGTCTTGTGACAATTTTTAG	GGAAGTCCTGGCGCTAATGA
PdPIP1;1	Podel.08G080400.1	CAAGAAGTGATGAYTGTGATGC	ACGTCACTGTTATAGCACGC
PdPIP1;2	Podel.03G137600.1	TTCAAGAGCAGAGCTTAATTTC	TTCAAAAAGCTAGATAATTTACAC
PdPIP1;3	Podel.10G194700.1	AAGAAGTGATTATTATGATTATGG	GATTTGAAGAAACACGTATTGCTA
PdPIP2;1	Podel.09G141800.1	TGCTAACATGGTGGCTCCC	GATCATCCCAGGCTTTCTTATC
PdPIP2;2	Podel.04G178700.1	TGGTACACTTGGCCACAATC	GCTAATGCTCCCACAAATGG
PdPIP2;3	Podel.16G093500.1	ATTCAGTTATGGAACGTACGC	AGCTCACAACCATGACATAAC
PdPIP2;4	Podel.10G227600.1	CCAACGGTTTTAAATCTCGGTTC	ACCGAAAGGGATAATAAAGGG
PdPIP2;5	Podel.06G138200.1	TGTGTACATTATGGTGTCGTG	CAAAGAAGCAGCACTACCTGAGAC
PdPIP2;6	Podel.08G048200.1	ATCCCAATCACAGGAACTGG	CTATGGCTGCACCAATGAAG
PdPIP2;7	Podel.09G012100.1	ATGACCATTGGCTCTTCTGG	GGCTTTAAGCATTGCTCCTG
PdPIP2;8	Podel.05G122300.1	GCAATCCTACTAGCTAAGGC	ACCCACATATCAAGTTGAGAC
PdPIP2;9	Podel.05G122200.1	ACTGGAACTGGCATCAATCC	CCAACCCAGAAAATCCACAG
PdPIP2;10	Podel.06G138300.1	CACAGTCGTGGTCAAGATGTC	ACTAGTTGATTATGAGATAGGGAG
PdTIP1;1	Podel.01G246100.1	TGCTGCCATTGTCTACGAGGTCAT	CACCACCACAGACGTGTCTTC
PdTIP1;2	Podel.09G026800.1	TGCTGCCATTGTCTACGAGGTCTG	AACAACACCAACATCATCCAAACC
PdTIP1;3	Podel.10G214800.1	GATTGGAAACTCCAGCTTTTGGAT	AATTTCCCTTCTTTGGATCCACT
PdTIP1;4	Podel.08G061500.1	ATTGGAAACACCAGCTTTTGGGC	AATTTCCCTTCTTCGGATCCACC
PdTIP1;5	Podel.16G102900.1	ATTGTGGGTGCTAACATTCTAGTC	CCACCAACCAGTGGTCCGGC
PdTIP1;6	Podel.06G131300.1	CAACCACTGGGTCTACTGGGCA	GAAAGAAAAAGCCGCGGCACTG
PdTIP1;7	Podel.09G003500.1	CGAGTTAATCTTCATCAGCCACAC	GGAAAAAATTCAACGTAGTCTTAAAC
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	TTCAGAAACTGGGGCAGGCGC
PdTIP2;4	Podel.01G168400.1	TCGCTTCAGGGATGAGTGCTGTT	TTCGGAAACTGGGGCAGCGGT
PdTIP3;1	Podel.18G157700.1	GAGCATTTGGGCCTGCTCTAG	GGTGAGCTACTGGCTCTGCTG
PdTIP3;2	Podel.17G170600.1	GAGCTTTTGGGCCTGCTTTAA	GGCGAGGTACTGGCTCTGTGG
PdTIP4;1	Podel.06G131300.1	ATGGGCTAGGTCCAATGCTGAC	GAAGAGGACGATGAGATCTTGTG

830 **References**

Aasamaa, K., Sober, A., 2001. Hydraulic conductance and stomatal sensitivity to changes of
leaf water status in six deciduous tree species. Biologia Plantarum 44, 65-73.

Aasamaa, K., Sober, A., Rahi, M., 2001. Leaf anatomical characteristics associated with
shoot hydraulic conductance, stomatal conductance and stomatal sensitivity to changes of
leaf water status in temperate deciduous trees. Australian Journal of Plant Physiology 28,
765-774.

- Anderberg, H.I., Kjellbom, P., Johanson, U., 2012. Annotation of *Selaginella moellendorffii*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
- responses of isohydric and anisohydric poplars to drought. J. Exp. Bot. 66, 4373-4381.
- Bartlett, M.K., Scoffoni, C., Sack, L., 2012. The determinants of leaf turgor loss point and
 prediction of drought tolerance of species and biomes: a global meta-analysis. Ecology Letter
 15, 393-405.
- Bartlett, M.K., Klein, T., Jansen, S., Choat, B., Sack, L., 2016. The correlations and
 sequence of plant stomatal, hydraulic, and wilting responses to drought. Proc Natl Acad Sci
 USA 113, 13098-13103.
- Begg, J.E., Turner, N.C., 1970. Water potential gradients in field tobacco. Plant Physiology46, 343-346.
- Ben Baaziz, K., Lopez, D., Rabot, A., Combes, D., Gousset, A., Bouzid, S. et al., 2012. Lightmediated K_{leaf} induction and contribution of both the PIP1s and PIP2s aquaporins in five tree species: walnut (*Juglans regia*) case study. Tree Physiol. 32, 423-434.
- Blackman, C.J., Brodribb, T.J., Jordan, G.J., 2009. Leaf hydraulics and drought stress:
 response, recovery and survivorship in four woody temperate plant species. Plant, Cell and
 Environment. 32, 1584-1595.
- Brodribb, T.J., Skelton, R.P., McAdam, S.A.M., Bienaimé, D., Lucani, C.J., Marmottant, P.,
- 2016a. Visual quantification of embolism reveals leaf vulnerability to hydraulic failure. NewPhytologist 209, 1403-1409.
- Brodribb, T.J., Bienaimé D., Marmottant, P., 2016b. Revealing catastrophic failure of leaf
 networks under stress. PNAS 113, 4865-4869.
- Brunel, N., Leduc, N., Poupard, P., Simoneau, P., Mauget, J.C., Viemont, J.D., 2002.
 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

- Buckley, T.N., John, G.P., Scoffoni, C., Sack, L., 2015. How does leaf anatomy in fluence water transport outside the xylem? Plant Physiology 168, 1616-1635.
- Bunce, J.A., 2006. How do leaf hydraulics limit stomatal conductance at high water vapourpressure 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
- from pine trees. Plant Mol. Biol. Rep. 11, 113-116.
- Chaumont, F., Tyerman, S.D., 2014. Aquaporins: highly regulated channels controlling plantwater relations. Plant Physiology 164, 1600-1618.
- Choat, B., Jansen, S., Brodribb, T.J., et al, 2012. Global convergence in the vulnerability offorests to drought. Nature 491, 752-755
- Czechowski, T., Stitt, M., Altmann, T., Udvardi, M.K. and Scheible, W.R., 2005 Genome-wide
 identification and testing of superior reference genes for transcript normalization in
 Arabidopsis. Plant Physiol. 139, 5–17.
- Banielson, J.A.H., Johanson, U., 2008. Unexpected complexity of the aquaporin gene family
 in the moss *Physcomitrella patens*. BMC Plant Biology 8, 45-60.
- Be Schepper, V., van Dusschoten, D., Copini, P., Jahnke, S., Steppe, K. 2012. MRI links
 stem water content to stem diameter variations in transpiring trees. Journal of Experimental
 Botany 63, 2645-2653.
- Bobra, J., Motyka, V., Dobrev, P., Malbeck, J., Prasil, I.T., Haisel, D., Gaudinova, A.,
 Havlova, M., Gubis, J., Vankova, R., 2010. Comparison of hormonal responses to heat,
 drought and combined stress in tobacco plants with elevated proline contents. Journal of
 Plant Physiology 167, 1360-1370.
- Fetter, K., Van Wilder, V., Moshelion, M., Chaumont, F., 2004. Interactions between plasma
 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
- and mesophyll conductance for CO₂. Plant Cell and Environment 35, 611-625.

- Fichot, R., Brignolas, F., Cochard, H., Ceulemans, R., 2015. Vulnerability to drought-induced
 cavitation in poplars: synthesis and future opportunities. Plant Cell Environ 38, 1233-1251.
- Garavillon-Tournayre, M., Gousset-Dupont, A., Gautier, F., Benoit, P., Conchon, P., Souchal,
 R., Lopez, D., Petel, G., Venisse, J.S., Bastien, C., Label, P., Fumanal, B., 2017. Integrated
 drought responses of black poplar: how important is phenotypic plasticity?, Physiologia
 Plantarum, 163, 30-44.
- Galmés, J., Pou, A., Alsina, M.M., Tomas, M., Medrano, H., Flexas, J., 2007. Aquaporin
 expression in response to different water stress intensities and recovery in Richter-110 (*Vitis*sp.): relationship with ecophysiological status. Planta 226, 671-681.
- Gattolin, S., Sorieul, M., Frigerio, L., 2011. Mapping of tonoplast intrinsic proteins in maturing
 and germinating *Arabidopsis* seeds reveals dual localization of embryonic TIPs to the
 tonoplast and plasma membrane. Molecular Plant. 4, 180-189.
- Griffiths, H., Weller, G., Toy, L.F.M., Dennis, R.J., 2013. You're so vein: Bundle Sheath
 physiology, phylogeny and evolution in C3 and C4 plants. Plant, Cell & Environment 36, 249261.
- Gupta, A.B., Sankararamakrishnan R., 2009. Genome-wide analysis of major intrinsic
 proteins in the tree plant *Populus trichocarpa*: characterization of XIP subfamily of
 aquaporins from evolutionary perspective. BMC Plant Biology 20, 134.
- Heckwolf, M., Pater, D., Hanson, D.T., Kaldenhoff, R., 2011. The *Arabidopsis thaliana*aquaporin *At*PIP1;2 is a physiologically relevant CO₂ transport facilitator. The Plant Journal
 67, 795-804.
- Heinen, R.B., Ye, Q., Chaumont, F., 2009. Role of aquaporins in leaf physiology. Journal ofExperimental Botany 60, 2971-2985.
- Heymann, J.B., Engel, A., 1999. Aquaporins: phylogeny, structure, and physiology of waterchannels. News Physiol Sci 14, 187-193.
- Hochberg, U., Bonel, A.G., David-Schwartz, R., Degu, A., Fait, A., Cochard, H., Peterlunger,
 E., Herrera, J.C., 2017. Grapevine acclimation to water deficit: the adjustment of stomatal
 and hydraulic conductance differs from petiole embolism vulnerability. Planta 245, 10911104.
- Hochberg, U., Windt, C.W., Ponomarenko, A., Zhang, Y.J., Gersony, J., Rockwell, E.F.,
 Holbrook, N.M., 2017. Stomatal Closure, Basal Leaf Embolism, and Shedding Protect the
 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.
- Josse, J., Husson, F., 2016. missMDA: A Package for Handling Missing Values in
 Multivariate Data. Analysis. Journal of Statistical Software, 70, 1-31.
- Kassambara, A., Mundt, F., 2017. factoextra: Extract and Visualize the Results of
 Multivariate Data Analyses. R package version 1.0.5. https://CRAN.Rproject.org/package=factoextra
- Whan, S.H., Ahmad, N., Ahmad, F., Kumar, R., 2010. Naturally Occurring Organic
 Osmolytes: From Cell Physiology to Disease Prevention IUBMB Life 62, 891-895.
- Koide, R.T., Robichaux, R.H., Morse, S.R., Smith, C.M., 1989. Plant water status, hydraulic
 resistance and capacitance. In Plant Physiological Ecology: Field Methods and
 Instrumentation (eds R.W. Pearcy, J.R. Ehleringer, H.A. Mooney & P.W. Rundel), pp. 161183. Chapman and Hall, New York, NY, USA.
- Larchevêque, M., Maurel, M., Desrochers A., Larocque, G.R., 2011. How does drought
 tolerance compare between two improved hybrids of balsam poplar and an unimproved
 native species? Tree Physiology 31, 240-249.
- Laur, J., Hacke, U.G., 2014. Exploring Picea glauca aquaporins in the context of needlewater uptake and xylem refilling. New Phytologist 203, 388-400.
- Le, S., Josse, J., HussonF., 2008. FactoMineR: An R Package for Multivariate Analysis.
 Journal of Statistical Software 25, 1-18.
- Lee, S.J., Murphy, C.T., Kenyon, C., 2009. Glucose shortens the life span of *C. elegans* by
 downregulating DAF-16/FOXO activity and aquaporin gene expression. Cell Metab. 10, 379391.
- Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25, 402-408.
- Liu, X., Bandyopadhyay, B., Nakamoto, T., Singh, B., Liedtke, W., Melvin, J.E., Ambudkar, I.
 2006. A role for AQP5 in activation of TRPV4 by hypotonicity: concerted involvement of
 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 Experimental962 Botany 63, 2217-2230.
- 963 Lopez, D., Venisse, J.S., Fumanal, B., Chaumont, F., Guillot, E., Daniels, M.J., Cochard, H.,
- Julien, J.L., Gousset-Dupont, A., 2013. Aquaporins and Leaf Hydraulics: Poplar Sheds NewLight. Plant Cell Physiology 54, 1963-1975.
- Lopez, D., Amira, M.B., Brown, D., Muries, B., Brunel-Michac, N., Bourgerie, S., Porcheron,
 B., Lemoine, R., Chrestin, H., Mollison, E., Di Cola, A., Frigerio, L., Julien, J.L., GoussetDupont, A., Fumanal, B., Label, P., Pujade-Renaud, V., Auguin, D., Venisse J.S., 2016. The *Hevea brasiliensis* XIP aquaporin subfamily: genomic, structural and functional
 characterizations with relevance to intensive latex harvesting. Plant Molecular Biology 91,
 375-396.
- Martin, C.E., Lin, T.C., Lin, K.C., Hsu, C.C., Chiou, W.L., 2004. Causes and consequences of
 high osmotic potentials in epiphytic higher plants. Journal of Plant Physiology 161, 11191124.
- Martin-StPaul, N., Delzon, S., Cochard, H., 2017. Plant resistance to drought depends on
 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.
- Maurel, C., Tacnet, F., Guclu, J., Guern, J., Ripoche, P., 1997. Purified vesicles of tobacco
 cell vacuolar and plasma membrane exhibit dramatically different water permeability and
 water channel activity. PNAS 94, 7103-7108.
- Nobel, P.S., 2006. Parenchyma-chlorenchyma water movement during drought for thehemiepiphytic cactus Hylocereus undatus. Annals of Botany 97, 469-474.
- Ohtsuka, A., Sack, L., Taneda, H., 2017. Bundle sheath lignification mediates the linkage of
 leaf hydraulics and venation. Plant Cell Environ. 18, 1-12.
- Pfaffl, M.W., Tichopad, A., Prgomet, C., Neuvians, T.P., 2004. Determination of stable
 housekeeping genes, differentially regu- lated target genes and sample integrity:
 BestKeeper–Excel-based tool using pair-wise correlations. Biotechnology Letter 26, 509-515.
- Pou, A., Medrano, H., Flexas, J., Tyerman, S.D., 2013. A putative role for TIP and PIP
 aquaporins in dynamics of leaf hydraulic and stomatal conductances in grapevine under
 water stress and re-watering. Plant Cell Environment 36, 828-843.
- Prado, K., Maurel, C., 2013. Regulation of leaf hydraulics: from molecular to whole plantlevels. Frontiers in Plant Science 4, 58-60.

- Prado, K., Boursiac, Y., Tournaire-Roux, C., Monneuse, J.M., Postaire, O., Da Ines, O.,
 Maurel, C., 2013. Regulation of *Arabidopsis* leaf hydraulics involves light-dependent
 phosphorylation of aquaporins in veins. Plant Cell 25, 1029-1039.
- R Core Team (2017). R: A language and environment for statistical computing. R Foundation
 for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.
- Rockwell, F.E., Holbrook, N.M., 2017. Leaf Hydraulic Architecture and Stomatal
 Conductance: A Functional Perspective. Plant Physiology 174, 1996-2007.
- Sack, L., Melcher, P.J., Zwieniecki, M.A., Holbrook, N.M., 2002. The hydraulic conductance
 of the angiosperm leaf lamina: a comparison of three measurement methods. Journal of
 Experimental Botany 53, 2177-2184.
- 1004 Sack, L., Holbrook, N.M., 2006. Leaf hydraulics. Annu. Rev. Plant Biol. 57, 361-381.
- Sack, L., Cowan, P.D., Jaikumar, N., Holbrook, N.M., 2003. The 'hydrology' of leaves: coordination of structure and function in temperate woody species. Plant Cell and Environment
 26, 1343-1356.
- 1008 Sack, L., Pasquet-Kok, J., 2011. http://www.publish.csiro.au/prometheuswiki/tiki-1009 pagehistory.php?page=Leaf pressure-volume curve parameters&preview=16.
- Sack, L., Scoffoni, C., 2013. Leaf venation: structure, function, development, evolution,
 ecology and applications in the past, present and future. New Phytologist 198, 983-1000.
- Sade, N., Shatil-Cohen, A., Attia, Z., Maurel, C., Boursiac, Y., Kelly, G., Granot, D., Yaaran,
 A., Lerner, S., Moshelion, M., 2014. The role of plasma membrane aquaporins in regulating
 the bundle sheath-mesophyll continuum and leaf hydraulics. Plant Physiology 166, 16091620.
- Sade, N., Moshelion, M. 2016. Plant aquaporins and abiotic stress. In Plant Aquaporins:
 From Transport to Signalling; Chaumont, F., Tyerman, S., Eds.; Spinger-Verlag: BerlinHeidelberg, Germany.
- Sakr, S., Alves, G., Morillon, R., Maurel, K., Decourteix, M., Guillot, A., Fleurat-Lessard, P.,
 Julien, J.L., Chrispeels, M.J., 2003. Plasma membrane aquaporins are involved in winter
 embolism recovery in walnut tree. Plant Physiology 133, 630-641.
- Schmidt, J.E., Kaiser, W.M., 1987. Response of the succulent leaves of *Peperomia 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
- a neotropical savanna: experimental manipulation and model simulations. Agric For Meteorol1026 150, 629–639

- Scoffoni, C., Jansen, S., 2016. I Can See Clearly Now Embolism in Leaves. Trends in PlantScience 21, 723-725.
- Scoffoni, C., Albuquerque, C., Brodersen, C.R., Townes, S.V., John, G.P., Cochard, H.,
 Buckley, T.N., McElrone, A.J., Sack, L., 2017a. Leaf vein xylem conduit diameter influences
 susceptibility to embolism and hydraulic decline. New Phytologist 213, 1076-1092.
- Scoffoni, C., Albuquerque, C., Brodersen, C.R., Townes, S.V., Grace, P.J., Bartlett, M.K.
 Buckley, T.N., McElrone, A.J., Sack, L., 2017b. Outside-Xylem Vulnerability, Not Xylem
 Embolism, Controls Leaf Hydraulic Decline during Dehydration. Plant Physiol, 173,19971210.
- Secchi, F., Maciver, B., Zeidel, M.L., Zwieniecki, M.A., 2009. Functional analysis of putative
 genes encoding the PIP2 water channel subfamily Populus trichocarpa. Tree Physiology 29,
 1467-1477.
- 1039 Secchi, F., Zwienecki, 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.
- Shatil-Cohen, A., Attia, Z. Moshelion, M., 2011. Bundle-sheath cell regulation of xylemmesophyll water transport via aquaporins under drought stress: a target of xylem-borne
 ABA? Plant J 67, 72-80.
- Smith, J.A.C., Schulte, P.J., Nobel, P.S., 1987. Water flow and water storage in *Agave 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.
- Steppe, K., De Pauw, D.J.W., Lemeur, R., Vanrolleghem, P.A. 2006. A mathematical model
 linking tree sap flow dynamics to daily stem diameter fluctuations and radial stem growth.
 Tree Physiology 26, 257-273.
- 1054 Terashima, I., 1992. Anatomy of non-uniform leaf photosynthesis. Photosynth Res. 31, 195-1055 212.
- Tissue, D.T., Yakir, D., Nobel, P.S., 1991. Diel water movement between parenchyma and
 chlorenchyma of two desert CAM plants under dry and wet conditions. Plant Cell and
 Environment 14, 407-413.

- Tyree, M.T., Hammel, H.T. 1972. The measurement of the turgor pressure and the water relations of plants by the pressure-bomb technique. J. Exp. Bot. 23, 267-282.
- Tyree, M.T., Alexander, J., Machado, J.L., 1992. Loss of hydraulic conductivity due to water
 stress in intact juveniles of *Quercus rubra* and *Populus deltoides*. Tree Physiology 10, 411415.
- 1064 van den Honert, T.H., 1948. Water transport in plants as a catenary process. Discuss1065 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 in1071 grapevine. Physiologia Plantarum. 158, 284-296.
- Warnes, G.R., Ben Bolker, W., Bonebakker, L., Gentleman, R., Liaw, W.H.A., Lumley, T.,
 Maechler, M., Magnusson, A., Moeller, S., Schwartz, M., Venables, B., 2016. gplots: Various
 R Programming Tools for Plotting Data. R package version 3.0.1.
- Wheeler, J.K., Huggett, B.A., Tofte, A.N., Rockwell, F.E., Holbrook, N.M., 2013. Cutting
 xylem under tension or supersaturated with gas can generate PLC and the appearance of
 rapid recovery from embolism. Plant, Cell and Environment 36, 1938-1949.
- Williams M.L., Thomas B.J., Farrar J.F., Pollock C.J., 1993. Visualizing the distribution of
 elements within Barley leaves by energy-dispersive x-ray image maps (Edx Maps). New
 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.
- Zelazny, E., Borst, J.W., Muylaert, M., Batoko, H., Hemminga, M.A., Chaumont, F., 2007.
 FRET imaging in living maize cells reveals that plasma membrane aquaporins interact to
- regulate their subcellular localization. Proc Natl Acad Sci USA 104, 12359-12364.