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Gene expression in vessel-associated cells upon xylem embolism repair in *Vitis vinifera* L. petioles.

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12 **Title: Gene expression in vessel-associated cells upon xylem embolism repair in**
13 ***Vitis vinifera* L. petioles**

14

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33

34

35 **Abstract**

36 In this work, the involvement of vessel-associated cells in embolism recovery was
37 investigated by studying leaf petiole hydraulics and expression profiles of aquaporins
38 and genes related to sugar metabolism.

39 Two different stress treatments were imposed onto grapevines to induce xylem
40 embolism: one involved a pressure collar applied to the stems, while the other consisted
41 of water deprivation (drought). Embolism formation and repair were monitored during
42 stress application and release (recovery). At the same time, stomatal conductance (g_s),
43 leaf water potential (Ψ_{leaf}), and leaf abscisic acid (ABA) concentration were measured.
44 For each treatment, gene transcript levels were assessed on vessel-associated cells
45 (isolated from leaf petioles by laser microdissection technique) and whole petioles.

46 Both treatments induced severe xylem embolism formation and drops in g_s and Ψ_{leaf} at a
47 lesser degree and with faster recovery in the case of application of the pressure collar.
48 Leaf ABA concentration only increased upon drought and subsequent recovery.
49 Transcripts linked to sugar mobilisation (encoding a β -amylase and a glucose-6-P
50 transporter) were over-expressed upon stress or recovery both in vessel-associated cells
51 and whole petioles. However, two aquaporin genes (*VvPIP2;1* and *VvPIP2;4N*) were
52 activated upon stress or recovery only in vessel-associated cells, suggesting a specific
53 effect on embolism refilling. Furthermore, the latter gene was only activated upon
54 drought and subsequent recovery, suggesting that either severe water stress or ABA are
55 required for its regulation.

56

57 **Keywords:** Aquaporins, Drought, Grapevine, Laser microdissection, Pressure collar,
58 Sugar metabolism.

59

60 **Abbreviations:** ABA (Abscisic acid), IRR (Irrigated), WS (Water stress), RWS
61 (Recovery from water stress), PC (Pressure collar), RPC (Recovery from pressure
62 collar), HCFM (Hydraulic Conductance Flow Meter), LMD (Laser MicroDissection),
63 RT-qPCR (Quantitative Real-Time PCR), VACs (Vessel Associated Cells).

64

65 **Introduction**

66

67 Vascular plants have evolved a long-distance transport system for water and minerals
68 through non-living xylem vessels. Long distance transport is driven by tension, as
69 postulated by the Cohesion-Tension theory (Tyree 2003) and further confirmed by
70 direct measurements of negative pressures in xylem (Angeles et al. 2004). Under high
71 tension (e.g., upon drought stress), water is metastable and, when gas-filled, xylem
72 vessels may become disrupted by breakage of water column continuity (cavitation), thus
73 causing embolism that drastically reduces the hydraulic conductance of xylem (in
74 grapevine, Schultz and Matthews 1988; Lovisolo and Schubert 1998; Tramontini et al.
75 2013). However, many aspects concerning the biophysics of embolism formation in
76 plants remain unclear (Clearwater and Goldstein 2005). Several studies suggested that
77 xylem cavitation is caused by environmental stress, such as drought (e.g., Tyree et al.
78 1994; Davis et al. 2002) and freezing temperatures (e.g., Just and Sauter 1991; Nardini
79 et al. 2000; Sakr et al. 2003). Nevertheless, cavitation is also a daily cyclical
80 phenomenon occurring even in well-watered plants (Holbrook et al. 2001; Lovisolo et
81 al. 2008; Zufferey et al. 2011).

82 Xylem embolisms can be refilled (recover) when xylem tension drops to values close to
83 zero. However, embolism recovery takes place also upon tension, and plant metabolism
84 plays an essential role in these conditions, as demonstrated by the effect of metabolic
85 inhibitors (Salleo et al. 1996, 2004; Lovisolo and Schubert 2006). Furthermore,
86 modifications of transcriptional profiles observed upon embolism recovery (Brodersen
87 et al. 2010, 2013; Secchi and Zwieniecki 2010, 2011; Perrone et al. 2012b) suggest that
88 plants can mount specific responses to xylem embolism. Different models have been
89 proposed to explain how plants induce an embolism-refilling process, most of which
90 include key roles for living parenchyma cells surrounding xylem vessels (Vessel-

91 Associated Cells: VACs). In these cells a decrease in starch content and an increase in
92 sucrose concentration are observed upon refilling (Salleo et al. 2009; Secchi and
93 Zwieniecki 2010, Nardini et al. 2011). Sucrose is probably translocated to adjacent
94 embolised vessels (Holbrook and Zwieniecki 1999; Tyree et al. 1999; Salleo et al. 2004;
95 Secchi et al. 2012), where it helps to establish an osmotic gradient that draws water into
96 the emboli by aquaporin-mediated transport. The involvement of starch hydrolysis and
97 water transport facilitators in the refilling process is supported by upregulation of genes
98 encoding β -amylases and plasma membrane intrinsic proteins (PIPs) in recovering
99 shoots of *Juglans regia*, *Populus trichocarpa*, and *Vitis vinifera* (Sakr et al. 2003;
100 Kaldenhoff et al. 2008; Secchi and Zwieniecki 2010, 2011; Perrone et al. 2012b).

101 Besides describing the molecular processes involved, a few studies have focused on the
102 signal transduction pathways induced by the presence of xylem embolism. Secchi et al.
103 (2011) investigated global gene expression responses in poplar subjected to artificial
104 cavitation, and they proposed a novel role for oxygen as a signal molecule acting in
105 parenchyma cells and triggering xylem refilling. In previous studies (Lovisollo et al.
106 2008; Perrone et al. 2012b), we reported high levels of ABA in petioles recovering from
107 embolism under high transpiration conditions, and we hypothesized an active role of
108 this hormone in triggering recovery processes. Thus, the metabolic *scenario* of
109 embolism recovery is still debated. Moreover, although it is supposed that most of these
110 metabolic reactions take place in VACs, this has never been proven directly due to
111 technical difficulty of isolating these cells.

112 The Laser MicroDissection (LMD) technique is a powerful tool to isolate cell
113 populations from heterogeneous tissues and offers the possibility of exploring transcript
114 profiles in specific cell types. LMD has successfully been used to study gene expression
115 in different plant tissues, such as epidermal cells, shoot meristem tissues, root cap
116 tissues and specific cells involved in plant-microbe interactions, such as those colonised

117 by arbuscular mycorrhizal or pathogenic fungi (Balestrini et al. 2009; Gomez and
118 Harrison 2009; Chandran et al. 2010; Giovannetti et al. 2012).

119 In this study, we induced xylem cavitation and subsequent recovery in grapevine leaf
120 petioles using two different techniques: one involved pressure application and release to
121 the stems, while the other consisted of water deprivation (drought) followed by
122 irrigation. We used LMD to dissect VACs from embolised petioles, and we profiled the
123 expression of genes involved in sugar metabolism and transport, as well as in water
124 transport facilitation, in both VAC and whole petiole samples. We demonstrate that
125 while some of the tested genes are activated by stress and subsequent recovery in whole
126 petioles, some aquaporin genes are exclusively expressed in VACs, supporting the
127 conclusion that the related proteins have a specific role in the embolism recovery
128 process.

129

130

131 **Materials and methods**

132

133 Plant material and experimental setup

134

135 Two-year-old *Vitis vinifera* L. cv. Grenache plants [Vivai Cooperativi Rauscedo-San
136 Giorgio della Richinvelda (PN), Italy] grafted onto *Vitis riparia* x *Vitis berlandieri*
137 420A were grown in a glasshouse under partially controlled climate conditions. The
138 temperature in the greenhouse was maintained in the 26–35°C range, and natural
139 light/night cycles were followed. Maximum photosynthetic photon flux density (PPFD)
140 ranged between 1330 and 1580 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Each plant grew in a 10-l pot filled with a
141 substrate composed of a sandy-loam soil (pH 7.0; available P 7.9 mg kg^{-1} ; organic
142 matter 1.37%; cation exchange capacity 4.58 meq 100 g^{-1})/expanded clay/peat mixture

143 (2:1:1 by weight). From budbreak (February, 10th) to the beginning of the experimental
144 period (August 1), plants were irrigated twice a week to maintain water container
145 capacity.

146 Treatments were applied during a period of high atmospheric evaporative water demand
147 in August (vapour pressure deficit averaging 25 mbar bar⁻¹). Among the 36 plants used
148 in this study, 24 were maintained at container capacity (Lovisolo and Schubert 1998):
149 50% of these plants were used as control (12 IRR replicate plants), and 50% were
150 subjected to artificial cavitation, imposed using a pressure collar (PC) treatment (12
151 replicate plants) followed by depressurisation (RPC). The remaining 12 plants were
152 subjected to water stress (WS) treatment followed by rehydration (RWS).
153 Measurements and tissue samples were taken on one experimental day. To allow the
154 collection of data from a sufficient number of replicates, plants were distributed among
155 four experimental days: in each of them, three randomly chosen IRR, three PC-RPC,
156 and three WS-RWS plants were subjected to analysis.

157 For the PC treatment, shoots of normally irrigated plants were exposed to positive
158 pressures, following the procedure reported by several authors (Salleo et al. 1996, 2004;
159 Tyree et al. 1999; Secchi and Zwieniecki 2010) with minor modifications. Our system
160 consisted of a narrow-diameter tube (diameter, 19.1 mm) sealed around the basal
161 internode of the shoot by using a custom-built holder and allowing the application of
162 pressure around the stem. During the experimental day, at 11:00 h, the pressure collar
163 was connected to a gaseous N₂ bomb to maintain a 2.7 MPa pressure for five hours.
164 Thereafter (at 16:00 h), the collar was removed in order to induce depressurisation and
165 recovery.

166 For the WS treatment, irrigation was withheld for a 10-d period prior to the
167 experimental day. This treatment induces cavitation in grapevine without producing
168 stress-related modifications of xylem development (Schultz and Matthews 1988;

169 Lovisolo and Schubert 1998; Lovisolo et al. 2008). Water-stressed plants were
170 rehydrated at 16:00 h of the experimental day by watering pots to container capacity.
171 For each experimental day, one replicate plant within each treatment was used for: i)
172 leaf gas exchange and xylem embolism analysis; ii) leaf water potential measurement
173 and iii) petiole and leaf sampling for LMD, and for gene expression on whole petioles
174 and ABA analysis.

175

176 Leaf gas exchange, leaf water potential and xylem embolism

177

178 Transpiration rate (E) and stomatal conductance (g_s) were measured on adult, non-
179 senescing leaves well exposed to direct sunlight [PPFD (400–700 nm) $\geq 1200 \mu\text{mol m}^{-2}$
180 s^{-1}], using an infrared gas analyser ADC-LCPro+ system (The Analytical Development
181 Company Ltd, Hoddesdon, UK). Measurements were taken on one leaf per plant at 30
182 min intervals between 10:00 and 19:00 h on each experimental day, and on IRR and
183 RWS plants also on the day after. Leaf water potential (Ψ_{leaf}) was assessed on one
184 transpiring leaf per plant and at each of the same time points by using a Scholander-type
185 pressure chamber (Soil Moisture Equipment Corp., Santa Barbara, CA, USA).

186 Xylem embolism extent was measured on leaf petioles, as previously described by
187 Lovisolo et al. (2008), using a Hydraulic Conductance Flow Meter (HCFM-XP,
188 Dynamax Inc., Houston, TX, USA) (Tyree et al. 1995). Measurements were made at
189 16:00 h of the experimental day for IRR, WS and PC treatments, at 19:00 h of the same
190 day for the RPC treatment, and at 19:00 h of the following day for the RWS treatment.
191 Briefly, one leaf petiole per plant was cut under water by bending the shoot and
192 submerging the petiole into a water container. Embolism extent was determined by
193 comparing the initial hydraulic conductivity (K_{hi}) with the maximum final hydraulic
194 conductivity (K_{hf}) recorded after a transient water flushing designed to eject the

195 embolism from the petiole. The intensity of embolism was expressed as the percentage
196 loss of conductivity (PLC) and calculated as $100 * (K_{hf} - K_{hi}) / K_{hf}$. Significant
197 differences among treatments were determined by applying a one-way ANOVA test
198 using the SPSS statistical software package (SPSS Inc., Cary, NC, USA, v.20).

199

200 Laser microdissection of vessel-associated cells, RT-PCR and semi-quantitative RT-
201 PCR analyses

202

203 Two petioles per plant were collected at the same time points of PLC determination.
204 They were cut into about 5-mm segments and immediately fixed in Farmer's solution
205 (EAA), containing 75% (v/v) ethanol and 25% (v/v) acetic acid (Kerk et al. 2003), then
206 stored overnight at 4°C for paraffin embedding. Farmer's solution was then removed
207 and petiole segments were dehydrated in a graded series (30-min steps) of ice-cold
208 ethanol (70%, 90% in sterile water and 100% [v/v] twice), followed by 100% Neoclear
209 (Merck, Darmstadt, Germany). The petiole segments were then gradually replaced with
210 paraffin (Paraplast plus; Sigma-Aldrich, St Louis, MO, USA), following the protocol
211 described by Balestrini et al. (2007). Petiole sections (12 µm) were cut using a rotary
212 microtome and transferred onto Leica RNase-free PEN foil slides (Leica Microsystem,
213 Inc., Bensheim, Germany) with sterile double-distilled water (ddH₂O, Elga LabWater,
214 Lane End Industrial Park, UK). Sections were dried at 40°C in a warming plate, stored
215 at 4°C, and used within 1 day.

216 A Leica AS LMD system was used to isolate cells from dried sections. Just before use,
217 the paraffin sections were deparaffinised by Neoclear treatment for 10 min and 100%
218 ethanol for 1 min, and then they were air-dried. The slides were placed face down on
219 the microscope. Laser parameters for dissection of selected cells were 40-XT objective

220 at power 45–55, and speed 4. The cells from each biological replicate were
221 subsequently collected (within one day) into a 0.5 ml RNase-free PCR tube.
222 After collection, 50 μ l of PicoPure RNA extraction buffer (Arcturus Engineering,
223 Mountain View, CA, USA) were added to each tube, followed by incubation at 42°C
224 for 30 min, centrifugation at 800 g for 2 min, and storage at –80°C. RNA was extracted
225 using the PicoPure kit (Arcturus Engineering). DNase treatment was not performed on
226 the kit column, as described in the kit protocol, but RNA was treated with Turbo DNase
227 after the extraction procedure (Applied Biosystems, Foster City, CA, USA), according
228 with the manufacturer’s instructions. RNA quality and quantity were checked using a
229 NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). A
230 One-Step RT-PCR kit (Qiagen, Valencia, CA, USA) was used. Reactions were carried
231 out in a final volume of 20 μ l, as previously described by Balestrini et al. (2007).
232 Samples were incubated at 50°C for 30 min, then at 95°C for 15 min. Amplification
233 reactions were run for 40 cycles: 94°C for 45 s, 58°C for 45 s and 72°C for 45 s. All
234 RT-PCR experiments were performed on at least two biological and two technical
235 replicates. RNA samples were checked for DNA contamination through RT-PCR
236 analyses conducted with the *VvEFL- α* specific primers. PCR products were separated on
237 a 1.9% agarose gel. Target genes and relative primer pairs are described in Table S1.
238 Semi-quantitative RT-PCR experiments were carried out in a final volume of 21 μ l
239 following the same protocol. Amplification reactions with specific primers for the
240 selected genes (Table S1) and control gene (*VvEFL- α*) were run for different cycles (35,
241 37, 40) to determine the exponential amplification phase, as previously reported by
242 Guether et al. (2009). For each step of semi-quantitative RT-PCR, 7 μ l of cDNA were
243 loaded on a 1.9% agarose gel.

244

245

246 Quantitative expression analysis on whole petioles and leaf ABA concentration

247

248 Expression changes of target transcripts were quantified on whole petiole samples by
249 quantitative real-time PCR (RT-qPCR). Two leaves per plant were collected at the same
250 time points of PLC determination. Petioles from each treatment were pooled,
251 immediately frozen in liquid nitrogen and stored at -80°C . Total RNA was extracted in
252 triplicate from the pooled samples following the protocol by Carra et al. (2007). RNA
253 integrity and quantity were checked using a 2100 Bioanalyser (Agilent, Santa Clara,
254 CA, USA). RNA samples were treated with DNase I, RNase-free (Fermentas: $50\text{ U }\mu\text{l}^{-1}$),
255 and first-strand cDNA was synthesised starting from $10\text{ }\mu\text{g}$ of total RNA by using the
256 High Capacity cDNA Reverse Transcription kit (Applied Biosystems) following the
257 manufacturers' instructions.

258 Reactions were carried out in a StepOnePlusTM RT-qPCR System (Applied Biosystems)
259 and the SYBR Green method (Power SYBR[®] Green PCR Master Mix, Applied
260 Biosystems) was used for quantifying amplification results (Perrone et al. 2012a). Three
261 technical replicates were run for each sample. Thermal cycling conditions were as
262 follows: an initial denaturation phase at 95°C for 10 min, followed by 40 cycles at 95°C
263 for 15 s and 60°C for 1 min (only for aquaporin primers a step at 56°C for 15 s was
264 added to the cycling stage). Specific annealing of primers was checked on dissociation
265 kinetics performed at the end of each RT-qPCR run. Expression of target transcripts
266 was quantified after normalisation to the geometric mean of the endogenous control
267 genes, *Ubiquitin* (*VvUBI*) and *Actin* (*VvACT1*). Gene expression data were calculated as
268 expression ratios (relative quantity, RQ) to IRR controls. Gene-specific primers are
269 listed in Table S1. Significant differences between treated and control samples were
270 investigated by applying a one-way ANOVA test ($P < 0.05$), using the SPSS statistical
271 software package (SPSS, v.20).

272 Leaf blades for ABA analysis were also immediately frozen at -80°C , and ABA
273 concentration was quantified following the method previously described by Lovisolo et
274 al. (2008).

275

276

277 **Results**

278

279 Leaf physiological parameters and leaf ABA concentration

280

281 Stem pressurisation obtained by application of a pressure collar induces xylem
282 embolism formation without imposing long-lasting stress on the organs located distally
283 to the collar. We compared the physiological responses (leaf gas exchange, petiole
284 xylem embolism, and petiole ABA concentration) induced by application of either the
285 pressure collar or water stress, and by the following depressurisation or rehydration.

286 As expected, application of the pressure collar effectively induced xylem embolism.
287 percentage loss of hydraulic conductivity (PLC) was about 10% in IRR petioles. WS
288 treatment induced an increase in PLC to about 80%, and application of the PC also
289 induced an increase in PLC to about 60%. Embolism recovery proceeded much faster in
290 RPC than in RWS petioles: upon irrigation of WS plants, PLC only decreased to 54%
291 after 27 h from the re-watering treatment, while stem depressurisation (RPC) allowed
292 almost full recovery from embolism within 3 h (10% PLC) (Fig. 1).

293 Application of the PC also induced water stress in the leaves, as shown by
294 measurements of leaf water potential (Ψ_{leaf}) and stomatal conductance (g_s), albeit at a
295 lower level than the application of WS. In irrigated (IRR) petioles, Ψ_{leaf} remained
296 relatively constant between -0.3 and -0.4 MPa. Ψ_{leaf} decreased to about -1.4 MPa upon
297 WS, and to about -1.2 MPa upon PC application (Fig. 2a). In IRR plants g_s averaged
298 $0.14 \text{ mol m}^{-2} \text{ s}^{-1}$ with an expected slight decrease in the afternoon. Leaf g_s was lower in
299 WS plants (less than $0.03 \text{ mol m}^{-2} \text{ s}^{-1}$) and in PC plants $0.06 \text{ mol m}^{-2} \text{ s}^{-1}$ (Fig. 2b). Leaf
300 transpiration rate (E) reflected the observed changes in g_s (Fig. 2c).

301 The behaviour of plants subjected to WS versus PC treatment was, however, quite
302 different when a recovery was induced by rehydration in the case of WS (RWS), and by

303 depressurisation in the case of PC (RPC). In RWS petioles, Ψ_{leaf} recovered very slowly,
304 reaching -0.5 MPa at 19:00 h the following day, while recovery upon depressurisation
305 was fast and complete, reaching -0.5 MPa at 19:00 h the following day, while recovery
306 upon depressurisation was fast and complete, reaching -0.5 MPa within 3 h (Fig. 2a).
307 Also, g_s and E recovery were slow in RWS plants, reaching about 50% of that of the
308 IRR controls at 19:00 h the following day, while, in RPC plants, g_s quickly (within 3 h)
309 recovered (Fig. 2b,c).

310 Since the maintenance of responses to water stress after rehydration in grapevine is
311 dependent on the persistence of stress-induced endogenous ABA, we reasoned that the
312 physiological differences observed between WS-RWS and PC-RPC plants could be due
313 to different intensities of an ABA signal. The ABA concentration in leaves of irrigated
314 plants did not differ from that measured in PC and RPC leaf samples, while in WS leaf
315 ABA was significantly higher, with values around 13 000 pmol g^{-1} DW. At the end of
316 recovery from water stress (RWS), ABA levels dropped to values comparable with
317 those of the IRR controls (Fig. 3).

318

319 Laser microdissection and analysis of gene expression in vessel-associated cells and
320 whole petioles

321

322 The LMD protocol preserved petiole vascular tissues fairly well, which allowed
323 identification of specific cell types, in particular VACs (present among xylem vessels)
324 and phloem (Fig. 4a,b). For each treatment, about 270–300 vascular cell groups were
325 obtained. RNA final concentrations ranged between 10 and 30 ng μl^{-1} , depending on
326 the sample type and on the number of collected vascular cell groups. RNA samples
327 from the LMD-isolated tissue were then used to study the expression of genes
328 putatively involved in xylem embolism formation and repair. More specifically, all

329 target transcripts were first analysed in VACs by carrying out one-step RT-PCR
330 experiments. In RT-plus reactions the presence of an amplified fragment of the expected
331 size (100 bp) was observed in all cell types tested, using specific primers for the
332 endogenous control gene *VvEF1- α* , while the absence of an amplified product in RT-
333 minus reactions excluded genomic DNA contamination (Fig. S1). Since for the majority
334 of the genes an expression signal was observed in several of the considered treatments
335 (data not shown), we further investigated transcript expression by semi-quantitative
336 PCR analysis after 35, 37 and 40 amplification cycles (Fig. 4). After 40 cycles, the
337 amplification had reached its plateau in all samples, whereas after 35 and 37 cycles it
338 was still in the exponential phase, thus allowing a semi-quantitative comparison of
339 transcript abundance. As shown in Fig. 4c, transcript abundance of the control gene
340 *VvEF1- α* was comparable in all samples. We analysed the expression of 12 genes
341 related to drought and ABA responses to sugar metabolism and to water transport,
342 which are regulated in grapevine petioles upon water stress and rehydration (Perrone et
343 al. 2012b). More specifically, we considered: three genes involved in sugar
344 metabolism—a plastidic glucose-6P transporter (*VvGPT1*), a sucrose transporter
345 (*VvSUC27*), and a plastidic β -amylase (*VvBAM3*); two genes encoding proteins
346 belonging to the LEA (Late Embryogenesis Abundant) family (*VvDHN1a* and
347 *VvLEA14*); three genes encoding components of signal transduction (*VvNAC72*,
348 *VvSnRK2.1*, and *VvCAL*) and activated by drought and ABA in several systems; and
349 four genes encoding PIP-type aquaporins—namely *VvPIP1;1*, *VvPIP1;2*, *VvPIP2;1*
350 (Vandeleur et al. 2009) and *VvPIP2;4N* (Perrone et al. 2012a). Expression analyses
351 performed on micro-dissected VACs showed that *VvGPT1* was activated by PC and
352 RPC treatments; *VvSUC27* expression was low in all treatments, while *VvBAM3* was
353 activated in WS- and PC-treated cells (Fig. 4d,f). Results on genes potentially tied to
354 signal transduction mechanisms showed that *VvSnRK2.1* was activated in WS petioles,

355 while *VvNAC72*, besides in WS cells, was also activated in PC and RPC samples (Fig.
356 4g,h); *VvCAL* transcripts were only detected in WS and RWS cells (Fig. 4k). Among
357 aquaporin genes, *VvPIP1;1* was one of the most highly expressed in embolism-inducing
358 treatments (WS and PC), but it was also activated in RWS and RPC cells (Fig. 4i).
359 *VvPIP1;2* expression was undetectable in all treatments (data not shown). *VvPIP2;1*
360 was more expressed in PC and RPC treatments than in the other treatments (Fig. 4j);
361 however, *VvPIP2;4N* transcripts were only observed in WS and mostly in RWS cells
362 (Fig. 4l). Considering the members of the LEA family, which are typically involved in
363 plant stress response, *VvDHN1a* was exclusively expressed in WS and PC cell samples,
364 whereas *VvLEA14* was mainly activated in PC and in RPC cells and to a lesser extent in
365 WS cells (Fig. 4m,n).

366 To verify the specificity of gene expression in VACs, quantitative real-time PCR (RT-
367 qPCR) experiments were performed on the same target genes working on whole petiole
368 samples. *VvGPT1* and *VvSUC27* expression followed the same patterns observed in
369 VACs (Fig. 5a,b). *VvBAM3* was significantly activated upon WS treatment (as in
370 VACs), but it was down-regulated in PC and RPC petioles, despite these latter
371 expression changes were not significant when compared to the IRR control (Fig. 5c).
372 The two *LEA* genes were both strongly up-regulated upon WS treatment and their
373 expression was still very high in RWS samples (Fig. 5d,e). Moreover, *VvLEA14* was
374 significantly over-expressed in PC petioles, mirroring the pattern observed in VACs
375 (Fig. 5d). No significant changes were observed for *VvDHN1a* transcripts in PC
376 samples compared to the IRR control (Fig. 5e), while the same gene was activated in
377 VACs upon this treatment.

378 In whole petiole, the expression of genes encoding components of signal transduction
379 followed patterns similar to those observed in VACs: both *VvNAC72* and *VvSnRK2;1*
380 levels increased in WS and RWS treatments (Fig. 5f,g), although the over-expression of

381 *VvSnRK2;1* was significant only in WS samples (Fig. 5g). Interestingly, *VvNAC72* was
382 also slightly activated in PC and RPC treatments (Fig. 5f), whereas
383 *VvSnRK2;1* transcripts underwent a significant down-regulation.

384 The same consideration can be made for *VvCAL* transcripts, which were highly
385 expressed in WS and RWS petioles, following the expression profile observed in VACs,
386 while they were significantly down-regulated in both PC and RPC samples (Fig. 5h).

387 Among aquaporin genes, *VvPIP1;1* was slightly activated in WS petioles and
388 significantly down-regulated in RWS, PC and RPC petioles (Fig. 6a), at variance with
389 the observations made in VACs; *VvPIP1;2* was up-regulated in all treatments compared
390 to the IRR control (Fig. 6b), whereas in all VAC samples the same gene was not
391 detected.

392 Finally, while the expression of *VvPIP2;1* followed the same pattern observed in VACs
393 (it strongly increased in PC and RPC), *VvPIP2;4N* transcriptional levels were
394 significantly down-regulated in all treatments (Fig. 6c,d).

395

396

397

398 **Discussion**

399

400 Induction of xylem embolism in grapevine by water stress and stem pressurisation

401

402 It is well known that water stress (and subsequent rehydration) can induce xylem
403 embolism formation and recovery. Nevertheless, this environmental condition triggers a
404 wide array of molecular changes, which can mask those strictly related to embolism
405 refilling processes. In order to control these masking effects, we employed, parallel to
406 water stress and rehydration, the technique of stem pressurisation/depressurisation to

407 induce embolism formation and repair with a limited incidence of other stress-induced
408 processes. Both water stress and stem pressurisation require petiole excision to assess
409 the degree of embolism, and this was reported to induce artefacts on *Acer rubrum* and
410 *Fraxinus americana* (Wheeler et al. 2013). However, direct observations of embolism
411 recovery obtained in the absence of petiole excision (Brodersen et al. 2010, 2013)
412 suggest that refilling in grapevine is not affected by such artefacts (Sperry, 2013).

413 In our experiment, stem pressurisation was obtained by applying a pressure collar to
414 grape stems. Other authors have already used artificial tools to induce xylem cavitation
415 in woody plants (e.g., Salleo et al. 1996; Mayr et al. 2006; Secchi and Zwieniecki
416 2011). These systems are particularly suited to increasing pressure gradients at air-water
417 interfaces into the plant organs, thus inducing embolism formation. The method we set
418 up avoids both air injection bores and wounding to create the air inlet. After about five
419 hours of PC treatment, PLC increased from 10% to 55% in petioles; following
420 depressurisation, PLC fully recovered within 3 hours.

421 The PC treatment was not devoid of effects on water potential and leaf gas exchange,
422 which decreased after pressurisation and recovered upon depressurisation. Nevertheless,
423 the time courses of PLC, water potential, and leaf gas exchange upon RPC and RWS
424 were clearly different, since RPC recovery kinetics were more rapid. Depressurised
425 twigs of laurel, previously submitted to a pressure collar treatment, showed faster and
426 larger xylem refilling than upon native embolism repair (Salleo et al. 1996). A slow
427 recovery of hydraulic conductance and transpiration after rehydration of drought-
428 exposed plants has been well documented in grapevine and linked to the persistence of
429 high ABA concentration after rehydration (Lovisolo et al. 2008; Flexas et al. 2009;
430 Zufferey et al. 2011; Perrone et al. 2012b). ‘Grenache’ is a drought-avoiding isohydric
431 grape genotype, particularly suited to study drought responses, since it is able to tolerate
432 long-term water stress conditions (Schultz 2003; Soar et al. 2004; Vandeleur et al. 2009)

433 through ABA-mediated control of stomatal closure (Lovisolo et al. 2008, 2010). In this
434 experiment, recovery in RPC plants was indeed associated with low leaf ABA
435 concentration.

436 Differences in leaf ABA concentration, and in the kinetics and intensity of leaf water
437 potential and gas exchange changes induced by the two types of treatment, likely reflect
438 diverse mechanisms of induction of xylem embolism. In the case of drought-induced
439 water stress, water status is negatively affected, and ABA concentration increases in the
440 leaves, inducing stomatal closure: increased PLC is thought to depend on the increased
441 xylem tension that develops as an effect of water potential changes. In the case of stem
442 pressurisation, no water loss takes place, and xylem embolism is likely the primary
443 effect, later followed by limitations of leaf water potential due to reduced xylem
444 hydraulic conductivity, and by stomatal closure. In this case, a reversible loss of leaf
445 hydraulic conductivity could be a means of amplifying the signal of evaporative
446 demand to the stomata in order to trigger the stomatal response, as suggested by
447 Brodribb and Holbrook (2004) and shown in grapevine by Zufferey et al. (2011).

448

449 Expression changes of genes putatively involved in embolism recovery

450

451 Embolism recovery is an active process, which requires energy and metabolic activity.
452 It takes place upon negative tensions in the xylem, and several mechanistic models have
453 been proposed to explain it. All these models converge in considering the pivotal role of
454 solutes, solute transporters, and water transport facilitators (aquaporins) in VACs.
455 Recently, two studies based on transcriptomic analysis of tissues undergoing embolism
456 recovery have reported some genes linked to these processes, which undergo significant
457 expression changes (Secchi et al. 2011; Perrone et al. 2012b). Nevertheless, these
458 studies have focused on whole tissues, where molecular processes localised in VACs

459 may not be detectable. To deepen the role of these genes, we have analysed the
460 expression of some of these transcripts in VACs isolated by LMD.

461 The importance of regulation of carbohydrate metabolism and transport in VACs during
462 the embolism recovery process has already been supported by physiological analyses
463 (Salleo et al. 1996; Nardini et al. 2011; Secchi et al. 2013) and by measurements of gene
464 expression changes (Secchi et al. 2011; Perrone et al. 2012b). *VvBAM3* encodes a beta-
465 amylase up-regulated by water stress (Perrone et al. 2012b) as its *Arabidopsis*
466 orthologue (Fulton et al. 2008). *VvGPT1* is annotated as a plastidic glucose-6P
467 symporter and is up-regulated upon embolism recovery (Perrone et al. 2012b). Its
468 closest *Arabidopsis* homologue, the Glc6P/phosphate translocator1 (*AtGPT1*), is
469 localised in vascular bundle sheath cells (Niewiadomski et al. 2005), where it
470 contributes to glucose-6-phosphate transport into plastids (Kunz et al. 2010). In grape
471 petioles, *VvGPT1* could operate in the reverse direction, providing a supply of GLU-6P
472 into the cytosol of VACs. *VvGPT1* and *VvBAM3* genes were both activated in VACs
473 upon the embolism-inducing treatments applied (WS and PC). This data is in agreement
474 with a picture of activated starch hydrolysis and GLU-6P export from plastids, which
475 provides soluble sugars required to support the embolism recovery process. *VvSUC27* is
476 an H⁺-dependent sucrose transporter, whose expression is associated with sink organs in
477 grape (Davies et al. 1999). In whole grape petioles, *VvSUC27* is down-regulated by all
478 treatments inducing embolism formation, and it has previously been observed to be also
479 down-regulated due to water stress (Perrone et al. 2012b). This suggests that, upon
480 xylem embolism, the main provision of sugars to VACs derives from starch breakdown
481 and not from phloem unloading. *VvSUC27* expression was almost absent in VACs,
482 where phloem cells are not present. However, the regulatory changes involving these
483 genes were not limited to VACs. Indeed, they were also detected in whole petioles,

484 suggesting that most of the petiole cells collaborate each other to the mobilisation of
485 soluble sugars that drives embolism recovery.

486 The picture was quite different in the case of aquaporins, which are thought to facilitate
487 water supply to the xylem, thus determining a successful refilling process (Kaldenhoff
488 et al. 2008). This hypothesis requires that the activation of these channels takes place in
489 the cells surrounding xylem vessels. Among the tested *PIP1* and *PIP2* genes, *VvPIP1;1*,
490 *VvPIP2;1* and *VvPIP2;4N* were expressed in VACs of either embolising or recovering
491 petioles, confirming a potential role for these proteins in embolism refilling. However,
492 in the case of *VvPIP1;1* and *VvPIP2;4N* genes, these expression differences were not
493 observed in whole petioles, both in this study and in a previous work by Perrone et al.
494 (2012b), suggesting that their activation was strictly localised in VACs. The role of
495 aquaporins in embolism refilling has been inferred from expression measurements
496 performed in different plants, such as olive (Secchi et al. 2007), grapevine (Galmés et
497 al. 2007), rice (Sakurai-Ishikawa et al. 2011), tobacco (Mahdieh et al. 2008), and poplar
498 (Secchi et al. 2011). Aquaporins could contribute to embolism refilling only indirectly,
499 by facilitating axial flow of water to the leaves and thus reducing the xylem tension
500 gradient. Our expression results, obtained for the first time at the VAC level, strengthen
501 the hypothesis that these aquaporins play a pivotal role in refilling xylem embolism.

502 On the contrary, *VvPIP2;1* follows a different model. Indeed, the activation of this gene
503 takes place both in VACs and whole petioles, suggesting that it is probably not directly
504 linked to either embolism formation or recovery, but it could indirectly contribute to the
505 process. Finally, *VvPIP1;2* was activated in petioles but not in VACs, and this points to
506 a dependency on stress but not to a role in embolism refilling.

507

508 Water-stress and pressure collar responses to xylem embolism

509

510 In PC plants, embolism induction and recovery were faster than in WS plants, and they
511 took place in the absence of an ABA confounding effect. Since ABA strictly controls
512 gene expression networks in plants and grapevine (Koyama et al. 2009), we thus
513 checked whether expression of genes induced by water stress and of genes involved in
514 embolism recovery could be affected by the two different treatments.

515 Two genes belonging to the late embryogenesis abundant (LEA) protein family,
516 encoding a LEA14 (*VvLEA14*) and a dehydrin (*VvDHN1a*), were tested. In *Arabidopsis*,
517 the *VvDHN1a* orthologue (AT1G07470) is activated by salt and cold stress, and by
518 ABA (Hundertmark et al. 2008). In grapevine, *VvDHN1a* expression is induced by
519 water stress (Cramer et al. 2007) and ABA (Koyama et al. 2009; Yang et al. 2012). Our
520 results show that both genes are activated upon WS in VACs and whole petiole
521 samples, as previously observed in cv. Cabernet Sauvignon by Cramer et al. (2007).
522 Nevertheless, in VACs these genes were also up-regulated upon PC treatment.

523 We further measured the expression of three stress-responsive genes involved in signal
524 transduction (*VvCAL*, *VvSnRK2;1*, *VvNAC72*). In detail, *VvCAL* is the grape orthologue
525 of the *AtCLM24* (AT5G37770) gene, which encodes a Ca²⁺ binding protein in response
526 to ABA stimulus, day length and salt stress (Delk et al. 2005). *VvSnRK2;1* encodes a
527 protein kinase involved in ABA signal transduction, strongly up-regulated in grape
528 leaves treated with exogenous ABA (Boneh et al. 2012). *VvNAC72* is the grape
529 orthologue of *AtNAC72* (AT4G27410), whose expression is strictly controlled by ABA
530 (Fujita et al. 2004); in grape petioles this gene is activated upon water stress (Perrone et
531 al. 2012b). Our results indicate that *VvCAL* is only activated in WS and RWS VACs,
532 and in WS whole petioles; *VvSnRK2;1* transcripts are more abundant upon WS both in
533 VACs and whole petioles, although a slight up-regulation of this gene could be
534 observed in VACs upon PC, RPC and RWS treatments. On the contrary, *VvNAC72* is
535 more expressed in PC and RPC VACs.

536 Such differences between the two treatments were also observed for genes putatively
537 related to embolism recovery. Expression of *VvPIP2;4N*, and, to a lesser extent of
538 *VvBAM3*, increased upon water stress. *VvPIP2;4N* is a root-specific grape aquaporin
539 (Perrone et al. 2012a) and the localisation of its expression in VACs, depending on
540 water stress, could explain the fact that this transcript is not detected in whole petioles.
541 In olive twigs it has been shown that *OePIP2;1* aquaporin expression is activated when
542 shoot hydraulic conductance recovers (Secchi et al. 2007), and generally there is an up-
543 regulation of aquaporin genes when rehydration also occurs in grapevine leaves
544 (Galmés et al. 2007) or petioles (Perrone et al. 2012b). In addition, a coupling of
545 aquaporin activation with an increment in leaf transpiration has also been reported in
546 rice roots, where transpiration demand triggers the up-regulation of PIPs localised both
547 at the proximal end of the endodermis and on the cell surface around xylem (Sakurai-
548 Ishikawa et al. 2011), and in drought-exposed/rehydrated tobacco roots (Mahdieh et al.
549 2008). An obvious candidate for gene activation exclusively under drought stress is a
550 surge in ABA concentration, and correspondingly we found no ABA increase in PC and
551 RPC-treated leaves. We have previously shown (Lovisololo et al. 2008; Perrone et al.
552 2012b) that, upon rehydration from water stress, grapevine leaves accumulate ABA at
553 levels even higher than during the stress itself, and this could be instrumental to
554 embolism recovery if contemporaneously VAC-specific aquaporins are activated as is
555 the case of *VvPIP2;4N*.

556 Other genes (*VvGPT1* and *VvPIP2;1*) are present only upon pressure collar
557 pressurisation and depressurisation. These treatments thus trigger embolism-induced
558 signals that are not induced in water-stressed plants, although embolism is also present
559 in the latter. An explanation for this apparently contradictory result can be found in the
560 different dynamics of embolism induction and recovery deriving from the two types of
561 treatment. These dynamics are much faster in PC and RPC treatments. This means that,

562 during PC and RPC treatments, a fast induction of embolism could elicit signals that are
563 not present when a slow induction of embolism occurs, such as the case of water stress
564 treatment. Secchi and Zwieniecki (2010), also using an artificial device to induce
565 formation of xylem embolism in poplar, proposed several possible signals evoked
566 during fast embolism induction, such as the accumulation of soluble sugars in the xylem
567 or oxidative stress. However, in natural (and agricultural) conditions, xylem embolism
568 almost invariably arises because of drought. The experimental use of devices, such as
569 the pressure collar, which is applied to obtain embolism in the absence of water stress,
570 could not be representative of this condition, since this condition seems to activate
571 genes that are not expressed by water stress and following recovery.

572

573

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579

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774 **Figure captions**

775

776 **Fig. 1** Percentage loss of hydraulic conductivity (PLC) measured on cv. Grenache
777 petioles. IRR, irrigated control; WS, water stress; RWS, recovery from water stress; PC,
778 pressure collar stress; RPC, recovery from pressure collar stress. Lower case letters
779 denote significant differences ($P < 0.05$) attested by using the Tukey's test, bars are
780 standard errors of the mean ($n = 4$)

781

782 **Fig. 2** Time course of daily changes in leaf water potential (Ψ_{leaf} , **a**), stomatal
783 conductance (g_s , **b**), and leaf transpiration (E , **c**), measured on cv. Grenache plants well
784 watered (IRR), subjected to water stress (WS) and pressure collar (PC) treatments. Gray
785 arrow displays the time of PC pressurisation and black arrow shows the time of both
786 WS re-watering and PC depressurisation, as described in Materials and Methods. Bars
787 are standard errors of the mean ($n = 4$). Boxes containing initials are positioned
788 according with the sampling time. IRR, irrigated control; WS, water stress; RWS,
789 recovery from water stress; PC, pressure collar stress; RPC, recovery from pressure
790 collar stress

791

792 **Fig. 3** ABA concentration ($\text{pmol g}^{-1} \text{DW}$) in leaves of cv. Grenache plants. Lower case
793 letters denote significant differences ($P < 0.05$) attested by using the Tukey's test; bars
794 are standard errors of the mean ($n = 4$)

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796

797 **Fig. 4** Microdissection of vessel associated cells around xylem vessels (target section
798 area is indicated with a black line) before **(a)** and after **(b)** laser cutting. The inset shows
799 collected cells; red arrows indicate vessel-associated cells. Pictures were taken using an
800 x40 objective lens; scale bars represent 50 μm . Xyl: xylem cells; Phl: phloem cells.

801 **c-j** Semi-quantitative RT-PCR analyses on micro-dissected cells using the elongation
802 factor gene (*VvEF1- α*) **(c)** as endogenous control. Numbers correspond to RT-PCR
803 cycles. **k-n** RT-PCR on micro-dissected cells using specific primers for *VvCAL* **(k)**,
804 *VvPIP2;4N* **(l)**, *VvDHN1a* **(m)** and *VvLEA14* **(n)** genes. The size of amplified sequences
805 is 100 bp. IRR, irrigated control; WS, water stress; RWS, recovery from water stress;
806 PC, pressure collar stress; RPC, recovery from pressure collar stress

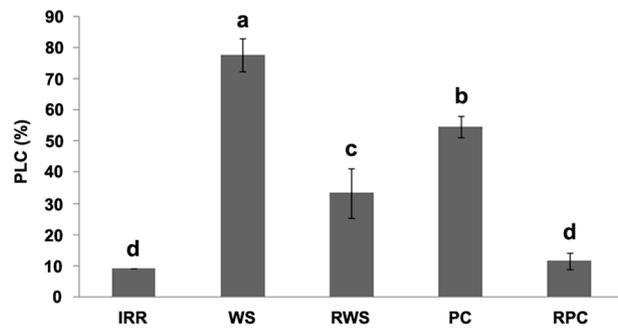
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808 **Fig. 5** Expression analysis of target genes in whole petioles: **a-h** RT-qPCR analyses on
809 cv. Grenache petioles for *VvGPT1* **(a)**, *VvSUC27* **(b)**, *VvBAM3* **(c)**, *VvLEA14* **(d)**,
810 *VvDHN1a* **(e)**, *VvNAC72* **(f)**, *VvSnRK2;1* **(g)** and *VvCAL* **(h)** transcripts. *Ubiquitin*
811 (*VvUBI*) and *Actin1* (*VvACT1*) were used as endogenous control genes for the
812 normalisation procedure. IRR, irrigated control; WS, water stress; RWS, recovery from
813 water stress; PC, pressure collar stress; RPC, recovery from pressure collar stress.
814 Lower case letters denote significant differences ($P < 0.05$) attested by using the
815 Tukey's test, bars are standard errors of the mean ($n = 3$).

816

817 **Fig. 6** Expression analysis of target aquaporin genes in whole petioles: **a-d** RT-qPCR
818 analyses on cv. Grenache petioles for *VvPIP1;1* **(a)**, *VvPIP1;2* **(b)**, *VvPIP2;1* **(c)** and
819 *VvPIP2;4N* **(d)** transcripts. *Ubiquitin* (*VvUBI*) and *Actin1* (*VvACT1*) were used as
820 endogenous control genes for the normalisation procedure. IRR, irrigated control; WS,
821 water stress; RWS, recovery from water stress; PC, pressure collar stress; RPC,

822 recovery from pressure collar stress. Lower case letters denote significant differences (P
823 < 0.05) attested by using the Tukey's test, bars are standard errors of the mean ($n = 3$).
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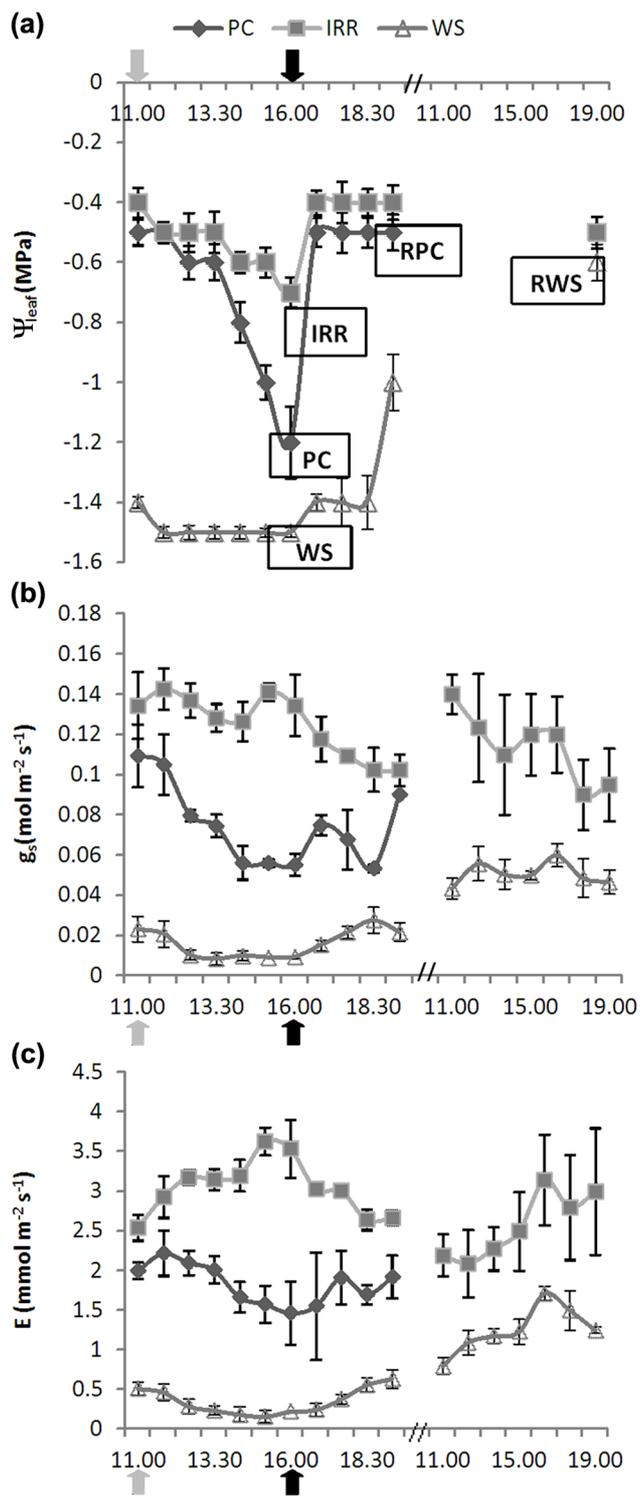


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828 Fig 1

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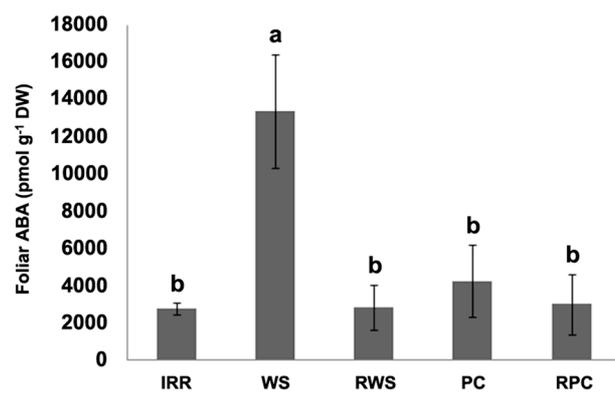


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831 Fig 2

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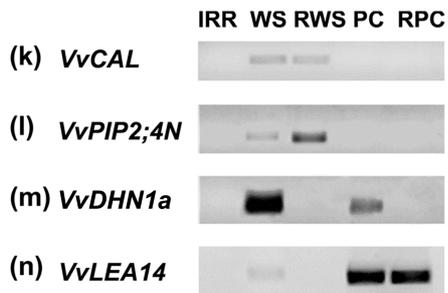
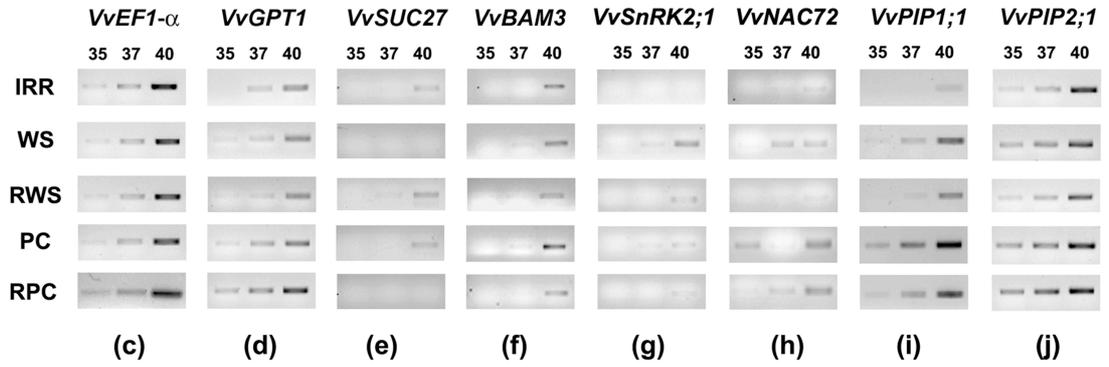
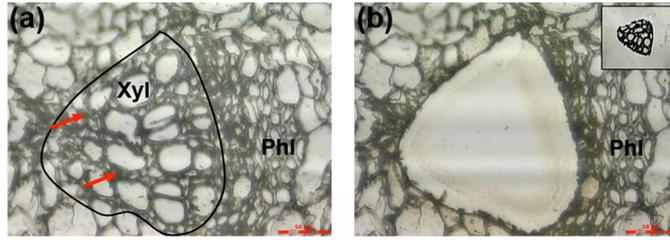
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835 Fig 3

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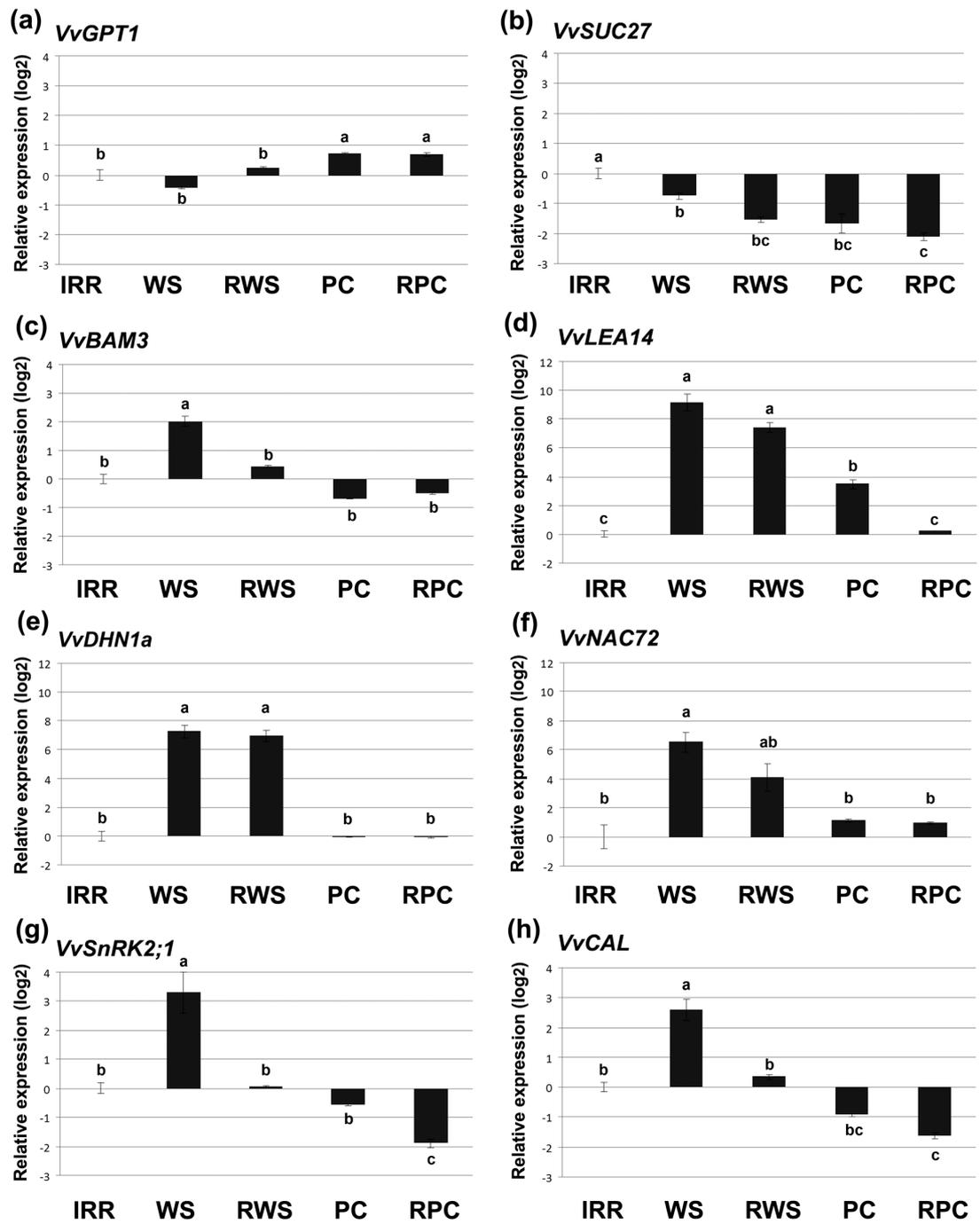
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840 Fig 4

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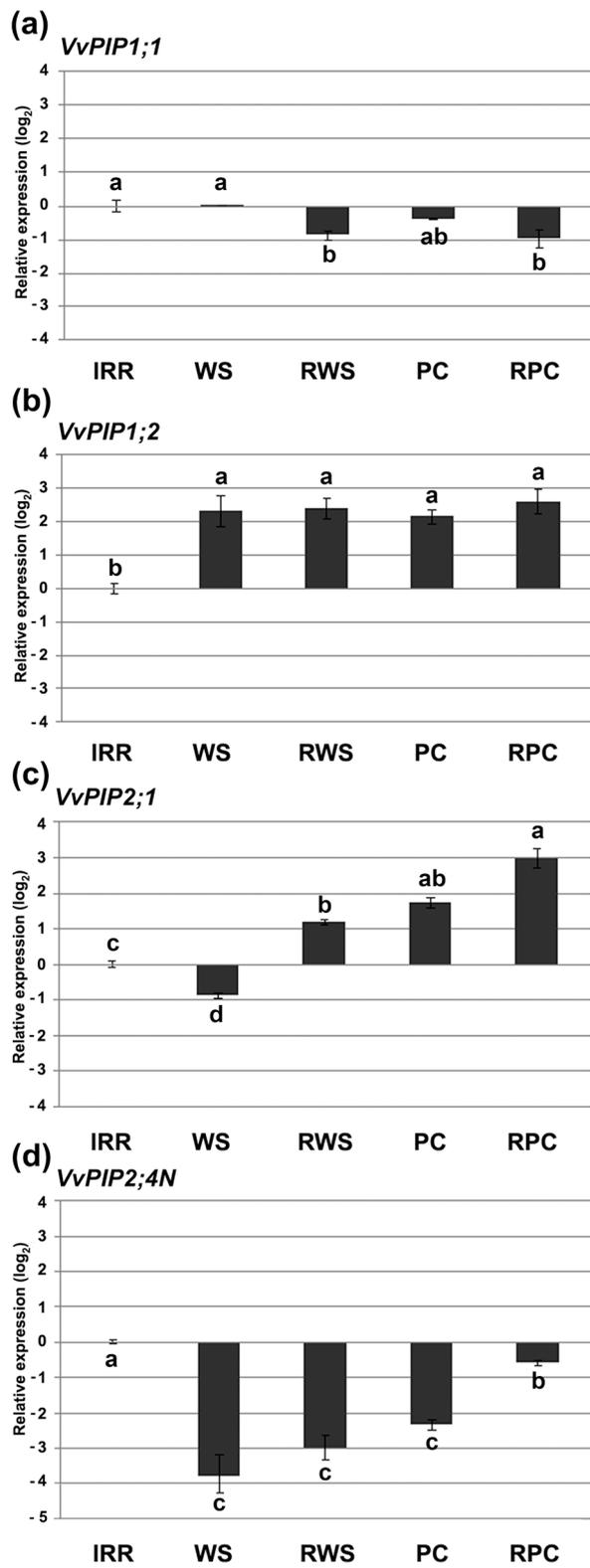


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843 Fig 5

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850 **Supplementary material captions**

851

852 **Supplementary Table S1** Oligonucleotides used in RT-PCR and semi-quantitative RT-
853 PCR on LMD samples, and in RT-qPCR on whole petiole samples

854

855 **Supplementary Fig. S1** RT-PCR analysis on micro-dissected samples using primers
856 for *VvEF1- α* gene as endogenous control gene. Any amplified product in RT-reactions
857 excludes DNA contaminations. IRR, irrigated control; WS, water stress; RWS, recovery
858 from water stress; PC, pressure collar stress; RPC, recovery from pressure collar stress

859

860 **Supplementary material**

861

862 **Table S1** Oligonucleotides used in RT-PCR and semi-quantitative RT-PCR on LMD samples, and
863 in RT-qPCR on whole petiole samples

Gene description	Gene ID (VVGDB 12X) and References	Primer	Primer sequences 5'-3'
<i>Actin 1 (VvACT1)</i>	VIT_04s0044g00580	Forward	GCCCCTCGTCTGTGACAATG
	Perrone <i>et al.</i> , 2012b	Reverse	CCTTGCCGACCCACAATA
<i>Ubiquitin (VvUBI)</i>	VIT_16s0098g01190	Forward	TCTGAGGCTTCGTGGTGGTA
	Perrone <i>et al.</i> , 2012b	Reverse	AGGCGTGCATAACATTTGCG
<i>Elongation factor 1-alpha (VvEF1-α)</i>	VIT_06s0004g03240	Forward	GAAGTGGGTGCTTGATAGGC
	Reid <i>et al.</i> , 2006	Reverse	AACCAAAATATCCGGAGTAAAAGA
<i>Dehydrin 1a (VvDHN1a)</i>	VIT_04s0023g02480	Forward	AACCCGGCGTGCTTCAT
	Perrone <i>et al.</i> , 2012b	Reverse	CATGCCCGGTATCCTCTCTTT
<i>Late Embryogenesis Abundant Protein 14 (VvLEA14)</i>	VIT_15s0046g02110	Forward	CGTACAACGCCAAGGTCTCA
	Perrone <i>et al.</i> , 2012b	Reverse	CATCTTCCCGACGCTATCA
<i>NAC domain-containing protein 72-like (VvNAC72)</i>	VIT_19s0014g03290	Forward	CGCCCTCCAATCTTCTTCTCT
	Perrone <i>et al.</i> , 2012b	Reverse	AGCTGTGAAAGCGGGTCAGT
<i>Serine threonine kinase 2.1 (VvSnRK2;1)</i>	VIT_02s0236g00130	Forward	AGATGTTTGGTCTTGTGGTGTGA
	Perrone <i>et al.</i> , 2012b	Reverse	CCCAATGGTCTTCCGGAAT
<i>Calmodulin-like protein (VvCAL)</i>	VIT_15s0048g00790	Forward	TGGTCAGAGAAGTGGACTGCAA
	Perrone <i>et al.</i> , 2012b	Reverse	CAGGTGCTGCTGCTACCAACT
<i>Beta amylase (VvBAM3)</i>	VIT_02s0012g00170	Forward	CTAGCAGCTGCCGAAGGAAT
	Perrone <i>et al.</i> , 2012b	Reverse	CAGCCGCATGAGACCTTGTT
<i>Glucose-6-phosphate transporter</i>	VIT_10s0116g00760	Forward	TTCCGGTGCCGGTCTACTT

<i>(VvGPT1)</i>	Perrone <i>et al.</i> , 2012b	Reverse	GCCCCATAAACCCAGTCAT
<i>Sucrose transporter (VvSUC27)</i>	VIT_18s0076g00250	Forward	TGACCCCCTACGTTAGCTT
	Perrone <i>et al.</i> , 2012b	Reverse	CCAACCTACCGGCTGCACAAT
<i>Aquaporin PIP1;1 (VvPIP 1;1)</i>	VIT_13s0067g00220	Forward	GAGTGGTGCTGGGCGTTGATC
	Choat <i>et al.</i> , 2009	Reverse	GTGGAATGCTACAGACATTAC
<i>Aquaporin PIP1;2 (VvPIP 1;2)</i>	VIT_15s0046g02420	Forward	TCCTCCATTTTCGTTTCTTC
	Choat <i>et al.</i> , 2009	Reverse	ATTGTAATAGAAGCAGCCCAG
<i>Aquaporin PIP2;1 (VvPIP 2;1)</i>	VIT_13s0019g04280	Forward	CCATTTTGATACCTTCTTCC
	Choat <i>et al.</i> , 2009	Reverse	TATCTACAATTCATGCCCTC
<i>Aquaporin PIP2;4N (VvPIP 2;4N)</i>	VIT_06s0004g02850	Forward	CTAGGATCTTTCAGGAGCAA
	Perrone <i>et al.</i> , 2012a	Reverse	TACTCCTCCACCATTGATGT

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869 **Fig. S1** RT-PCR analysis on microdissected samples using primers for *VvEF1-α* gene, as
870 housekeeping gene. Any amplified product in RT- reactions excludes DNA contaminations. IRR,
871 irrigated control; WS, water stress; RWS, recovery from water stress; PC, pressure collar stress;
872 RPC, recovery from pressure collar stress.

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