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ORIGINAL ARTICLE

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

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Abstract In this work, the involvement of vessel-associated cells in embolism recovery was investigated by studying leaf petiole hydraulics and expression profiles of aquaporins and genes related to sugar metabolism. Two different stress treatments were imposed onto grapevines to induce xylem embolism: one involved a pressure collar applied to the stems, while the other consisted of water deprivation (drought). Embolism formation and repair were monitored during stress application and release (recovery). At the same time, stomatal conductance (g_s), leaf water potential (Ψ_{leaf}) and leaf abscisic acid (ABA) concentration were measured. For each treatment, gene transcript levels were assessed on vessel-associated cells (isolated from leaf petioles by laser microdissection technique) and whole petioles. Both treatments induced severe xylem embolism formation and drops in g_s and Ψ_{leaf} at a lesser degree and with faster recovery in the case of application of the pressure collar. Leaf ABA concentration only increased upon drought and subsequent recovery. Transcripts linked to sugar mobilisation (encoding a β-amylase and a glucose-6-P transporter) were over-expressed upon stress or recovery, both in vessel-associated cells and whole petioles. However, two aquaporin genes (*VvPIP2;1* and *VvPIP2;4N*) were activated upon stress or recovery only in vessel-associated cells, suggesting a specific effect on embolism refilling. Furthermore, the latter gene was only activated upon drought and subsequent recovery, suggesting that either severe water stress or ABA is required for its regulation.

Keywords Aquaporins · Drought · Grapevine · Laser microdissection · Pressure collar · Sugar metabolism

Abbreviations

ABA	Abscisic acid
IRR	Irrigated
WS	Water stress
RWS	Recovery from water stress
PC	Pressure collar
RPC	Recovery from pressure collar
HCFM	Hydraulic conductance flow meter
LMD	Laser microdissection
RT-qPCR	Quantitative real-time PCR
VACs	Vessel-associated cells

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Introduction

Vascular plants have evolved a long-distance transport system for water and minerals through non-living xylem

vessels. Long-distance transport is driven by tension, as postulated by the cohesion-tension theory (Tyree 2003) and further confirmed by direct measurements of negative pressures in xylem (Angeles et al. 2004). Under high tension (e.g. upon drought stress), water is metastable and, when gas filled, xylem vessels may become disrupted by breakage of water column continuity (cavitation), thus causing embolism that drastically reduces the hydraulic conductance of xylem (in grapevine, Schultz and Matthews 1988; Lovisolo and Schubert 1998; Tramontini et al. 2013). However, many aspects concerning the biophysics of embolism formation in plants remain unclear (Clearwater and Goldstein 2005). Several studies suggest that xylem cavitation is caused by environmental stress, such as drought (e.g. Tyree et al. 1994; Davis et al. 2002) and freezing temperatures (e.g. Just and Sauter 1991; Nardini et al. 2000; Sakr et al. 2003). Nevertheless, cavitation is also a daily cyclical phenomenon occurring even in well-watered plants (Holbrook et al. 2001; Lovisolo et al. 2008; Zufferey et al. 2011).

Xylem embolisms can be refilled (recover) when xylem tension drops to values close to zero. However, embolism recovery takes place also upon tension, and plant metabolism plays an essential role in these conditions, as demonstrated by the effect of metabolic inhibitors (Salleo et al. 1996, 2004; Lovisolo and Schubert 2006). Furthermore, modifications of transcriptional profiles observed upon embolism recovery (Brodersen et al. 2010, 2013; Secchi and Zwieniecki 2010, 2011; Perrone et al. 2012b) suggest that plants can mount specific responses to xylem embolism. Different models have been proposed to explain how plants induce an embolism refilling process, most of which include key roles for living parenchyma cells surrounding xylem vessels (vessel-associated cells: VACs). In these cells a decrease in starch content and an increase in sucrose concentration are observed upon refilling (Salleo et al. 2009; Secchi and Zwieniecki 2010; Nardini et al. 2011). Sucrose is probably translocated to adjacent embolised vessels (Holbrook and Zwieniecki 1999; Tyree et al. 1999; Salleo et al. 2004; Secchi and Zwieniecki 2012), where it helps to establish an osmotic gradient that draws water into the emboli by aquaporin-mediated transport. The involvement of starch hydrolysis and water transport facilitators in the refilling process is supported by up-regulation of genes encoding β-amylases and plasma membrane intrinsic proteins (PIPs) in recovering shoots of *Juglans regia*, *Populus trichocarpa* and *Vitis vinifera* (Sakr et al. 2003; Kaldenhoff et al. 2008; Secchi and Zwieniecki 2010, 2011; Perrone et al. 2012b).

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

The laser microdissection (LMD) technique is a powerful tool to isolate cell populations from heterogeneous tissues and offers the possibility of exploring transcript profiles in specific cell types. LMD has successfully been used to study gene expression in different plant tissues, such as epidermal cells, shoot meristem tissues, root cap tissues and specific cells involved in plant–microbe interactions, such as those colonised by arbuscular mycorrhizal or pathogenic fungi (Balestrini et al. 2009; Gomez and Harrison 2009; Chandran et al. 2010; Giovannetti et al. 2012).

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

Materials and methods

Plant material and experimental setup

Two-year-old *V. vinifera* L. cv. Grenache plants [Vivai Cooperativi Rauscedo-San Giorgio della Richinvelda (PN), Italy] grafted onto *Vitis riparia* × *Vitis berlandieri* 420A were grown in a glasshouse under partially controlled climate conditions. The temperature in the greenhouse was maintained in the 26–35 °C range and natural light/night cycles were followed. Maximum photosynthetic photon flux density (PPFD) ranged between 1,330 and 1,580 µmol m^{-2} s^{-1}. Each plant grew in a 10-l pot filled with a substrate composed of a sandy-loam soil (pH 7.0; available *P* 7.9 mg kg^{-1}; organic matter 1.37 %; cation exchange capacity 4.58 meq 100 g^{-1})/expanded clay/peat

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

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

For the PC treatment, shoots of normally irrigated plants were exposed to positive pressures, following the procedure reported by several authors (Salleo et al. 1996, 2004; Tyree et al. 1999; Secchi and Zwieniecki 2010) with minor modifications. Our system consisted of a narrow-diameter tube (diameter 19.1 mm) sealed around the basal internode of the shoot by using a custom-built holder and allowing the application of pressure around the stem. During the experimental day, at 11:00 h, the pressure collar was connected to a gaseous N_2 bomb to maintain a 2.7 MPa pressure for 5 h. Thereafter (at 16:00 h), the collar was removed to induce depressurisation and recovery.

For the WS treatment, irrigation was withheld for a 10-day period prior to the experimental day. This treatment induces cavitation in grapevine without producing stress-related modifications of xylem development (Schultz and Matthews 1988; Lovisolo and Schubert 1998; Lovisolo et al. 2008). Water-stressed plants were rehydrated at 16:00 h of the experimental day by watering pots to container capacity.

For each experimental day, one replicate plant within each treatment was used for: (1) leaf gas exchange and xylem embolism analysis; (2) leaf water potential measurement and (3) petiole and leaf sampling for LMD and for gene expression on whole petioles and ABA analysis.

Leaf gas exchange, leaf water potential and xylem embolism

Transpiration rate (E) and stomatal conductance (g_s) were measured on adult, non-senescing leaves well exposed to direct sunlight [PPFD (400–700 nm) $\geq$ 1,200 μmol m^{-2} s^{-1}], using an infrared gas analyser ADC-LCPro+ system (The Analytical Development Company Ltd, Hoddesdon, UK). Measurements were taken on one leaf per plant at 30 min intervals between 10:00 and 19:00 h on each experimental day, and on IRR and RWS plants also on the day after. Leaf water potential (Ψ_{leaf}) was assessed on one transpiring leaf per plant and at each of the same time points using a Scholander-type pressure chamber (Soil Moisture Equipment Corp., Santa Barbara, CA, USA).

Xylem embolism extent was measured on leaf petioles, as previously described by Lovisolo et al. (2008), using a hydraulic conductance flow meter (HCFM-XP, Dynamax Inc., Houston, TX, USA) (Tyree et al. 1995). Measurements were made at 16:00 h of the experimental day for IRR, WS and PC treatments, at 19:00 h of the same day for the RPC treatment and at 19:00 h of the following day for the RWS treatment. Briefly, one leaf petiole per plant was cut under water by bending the shoot and submerging the petiole into a water container. Embolism extent was determined by comparing the initial hydraulic conductivity (K_{hi}) with the maximum final hydraulic conductivity (K_{hf}) recorded after a transient water flushing designed to eject the embolism from the petiole. The intensity of embolism was expressed as the percentage loss of conductivity (PLC) and calculated as $100 \times (K_{hf} - K_{hi})/K_{hf}$. Significant differences among treatments were determined by applying a one-way ANOVA test using the SPSS statistical software package (SPSS Inc., Cary, NC, USA, v.20).

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

Two petioles per plant were collected at the same time points of PLC determination. They were cut into about 5-mm segments and immediately fixed in Farmer's solution (EAA), containing 75 % (v/v) ethanol and 25 % (v/v) acetic acid (Kerk et al. 2003), then stored overnight at 4 °C for paraffin embedding. Farmer's solution was then removed, and petiole segments were dehydrated in a graded series (30-min steps) of ice-cold ethanol (70, 90 % in sterile water and 100 % [v/v] twice), followed by 100 % Neo-clear (Merck, Darmstadt, Germany). The petiole segments were then gradually replaced with paraffin (Paraplast plus; Sigma-Aldrich, St Louis, MO, USA), following the protocol described by Balestrini et al. (2007). Petiole sections (12 μm) were cut using a rotary microtome and transferred onto Leica RNase-free PEN foil slides (Leica Microsystem, Inc., Bensheim, Germany) with sterile double-distilled water (ddH_2O, Elga LabWater, Lane End Industrial Park, UK). Sections were dried at 40 °C in a warming plate, stored at 4 °C and used within 1 day.

A Leica AS LMD system was used to isolate cells from dried sections. Just before use, the paraffin sections were

deparaffinised by Neoclear treatment for 10 min and 100 % ethanol for 1 min and then air dried. The slides were placed face down on the microscope. Laser parameters for dissection of selected cells were 40-XT objective at power 45–55 and speed 4. The cells from each biological replicate were subsequently collected (within 1 day) into a 0.5-ml RNase-free PCR tube.

After collection, 50 μl of PicoPure RNA extraction buffer (Arcturus Engineering, Mountain View, CA, USA) was added to each tube, followed by incubation at 42 °C for 30 min, centrifugation at 800*g* for 2 min and storage at −80 °C. RNA was extracted using the PicoPure kit (Arcturus Engineering). DNase treatment was not performed on the kit column, as described in the kit protocol, but RNA was treated with Turbo DNase after the extraction procedure (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. RNA quality and quantity were checked using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). A One-Step RT-PCR Kit (Qiagen, Valencia, CA, USA) was used. Reactions were carried out in a final volume of 20 μl, as previously described by Balestrini et al. (2007). Samples were incubated at 50 °C for 30 min, then at 95 °C for 15 min. Amplification reactions were run for 40 cycles: 94 °C for 45 s, 58 °C for 45 s and 72 °C for 45 s. All RT-PCR experiments were performed on at least two biological and two technical replicates. RNA samples were checked for DNA contamination through RT-PCR analyses conducted with the *VvEF1-α* specific primers. PCR products were separated on a 1.9 % agarose gel. Target genes and relative primer pairs are described in Table S1.

Semi-quantitative RT-PCR experiments were carried out in a final volume of 21 μl following the same protocol. Amplification reactions with specific primers for the selected genes (Table S1) and control gene (*VvEF1-α*) were run for different cycles (35, 37, 40) to determine the exponential amplification phase, as previously reported by Guether et al. (2009). For each step of semi-quantitative RT-PCR, 7 μl of cDNA was loaded on a 1.9 % agarose gel.

Quantitative expression analysis on whole petioles and leaf ABA concentration

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

Reactions were carried out in a StepOnePlus™ RT-qPCR System (Applied Biosystems), and the SYBR Green method (Power SYBR® Green PCR Master Mix, Applied Biosystems) was used for quantifying amplification results (Perrone et al. 2012a). Three technical replicates were run for each sample. Thermal cycling conditions were as follows: an initial denaturation phase at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min (only for aquaporin primers a step at 56 °C for 15 s was added to the cycling stage). Specific annealing of primers was checked on dissociation kinetics performed at the end of each RT-qPCR run. Expression of target transcripts was quantified after normalisation to the geometric mean of the endogenous control genes, *Ubiquitin* (*VvUBI*) and *Actin* (*VvACT1*). Gene expression data were calculated as expression ratios (relative quantity, RQ) to IRR controls. Gene-specific primers are listed in Table S1. Significant differences among treatments were statistically analysed by applying a one-way ANOVA test and the Tukey's post hoc test was used for means separation when ANOVA results were significant. The SPSS statistical software package (SPSS, v.20) was used to run statistical analyses.

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

Results

Leaf physiological parameters and leaf ABA concentration

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

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

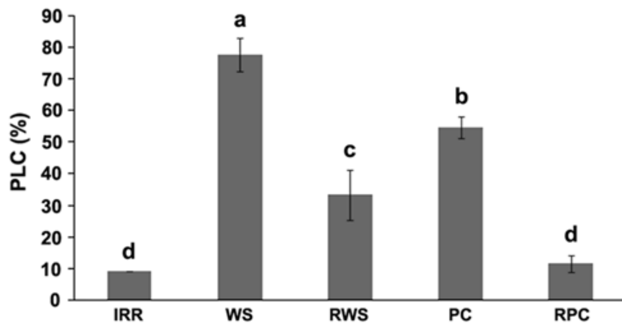


Fig. 1 Percentage loss of hydraulic conductivity (PLC) measured on cv. Grenache petioles. *IRR* irrigated control, *WS* water stress, *RWS* recovery from water stress, *PC* pressure collar stress, *RPC* recovery from pressure collar stress. Different *lowercase letters* denote significant differences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

almost full recovery from embolism within 3 h (10 % PLC) (Fig. 1).

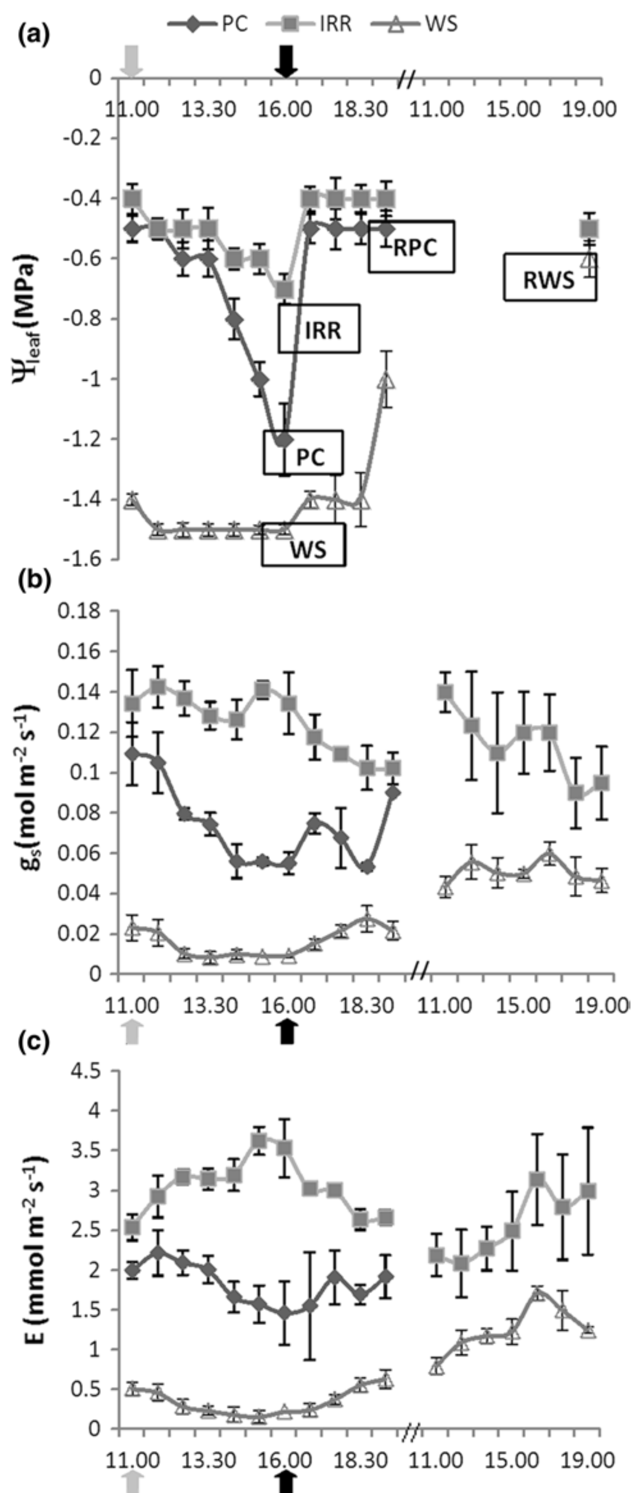
Application of the PC also induced water stress in the leaves, as shown by measurements of leaf water potential (Ψ_{leaf}) and stomatal conductance (g_s), albeit at a lower level than the application of WS. In irrigated (IRR) petioles, Ψ_{leaf} remained relatively constant between −0.3 and −0.4 MPa.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

The behaviour of plants subjected to WS versus PC treatment was, however, quite different when recovery was induced by rehydration in the case of WS (RWS) and by depressurisation in the case of PC (RPC). In RWS petioles, Ψ_{leaf} recovered very slowly, reaching −0.5 MPa at 19:00 h the following day, while recovery upon depressurisation was fast and complete, reaching −0.5 MPa within 3 h (Fig. 2a). Also, g_s and E recovery were slow in RWS plants, reaching about 50 % of that of the IRR controls at 19:00 h the following day, while in RPC plants g_s quickly (within 3 h) recovered (Fig. 2b, c).

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

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

The LMD protocol preserved petiole vascular tissues fairly well, which allowed identification of specific cell types, in particular VACs (present among xylem vessels) and phloem (Fig. 4a, b). For each treatment, about 270–300 vascular cell groups were obtained. RNA final concentrations ranged between 10 and 30 ng μl^{-1}, depending on the sample type and on the number of collected vascular cell groups. RNA samples from the LMD-isolated tissue were then used to study the expression of genes putatively involved in xylem embolism formation and repair. More specifically, all target transcripts were first analysed in VACs by carrying out one-step RT-PCR experiments. In RT-plus reactions the presence of an amplified fragment of the expected size (100 bp) was observed in all cell types tested, using specific primers for the endogenous control gene *VvEF1-α*, while the absence of an amplified product in RT-minus reactions excluded genomic DNA contamination (Fig. S1). Since for the majority of the genes an expression signal was observed in several of the considered treatments (data not shown), we further investigated transcript expression by semi-quantitative PCR analysis after 35, 37 and 40 amplification cycles (Fig. 4). After 40 cycles, the amplification had reached its plateau in all samples, whereas after 35 and 37 cycles it was still in the exponential phase, thus allowing a semi-quantitative comparison of transcript abundance. As shown in Fig. 4c, transcript abundance of the control gene *VvEF1-α* was comparable in all samples. We analysed the expression of 12 genes related to drought and ABA responses, sugar metabolism and water transport, which are regulated in grapevine petioles upon water stress and rehydration (Perrone et al. 2012b). More specifically, we considered three genes involved in sugar metabolism, a plastidic glucose-6P transporter (*VvGPT1*), a sucrose transporter (*VvSUC27*) and a plastidic β-amylase (*VvBAM3*); two genes encoding proteins belonging to the LEA (Late Embryogenesis Abundant) family (*VvDHN1a* and *VvLEA14*); three genes encoding components of signal transduction (*VvNAC72, VvSnRK2.1,* and *VvCAL*) and activated by drought and ABA in several systems; and four genes encoding PIP-type aquaporins, namely *VvPIP1;1*, *VvPIP1;2*, *VvPIP2;1* (Vandeleur et al. 2009) and *VvPIP2;4N* (Perrone et al. 2012a). Expression analyses performed on microdissected VACs showed that *VvGPT1* was activated by PC and RPC treatments; *VvSUC27* expression was low in all treatments, while *VvBAM3* was activated in WS- and PC-treated cells (Fig. 4d, f). Results on genes potentially tied to signal transduction mechanisms



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

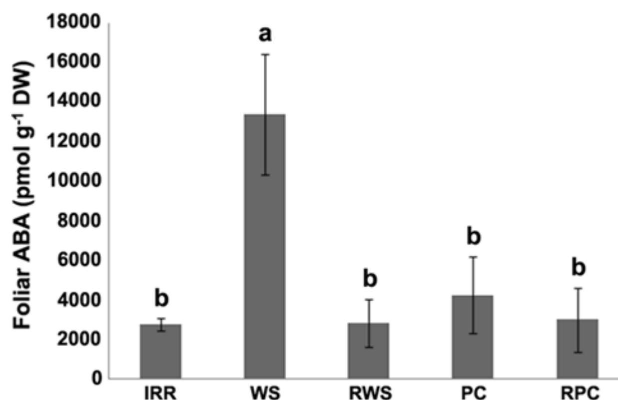


Fig. 3 ABA concentration (pmol g^{-1} DW) in leaves of cv. Grenache plants. Different *lowercase letters* denote significant differences ($P < 0.05$) attested using the Tukey's test; *bars* are standard errors of the mean ($n = 4$)

showed that *VvSnRK2.1* was activated in WS petioles, while *VvNAC72* was activated in WS cells, as well as in PC and RPC samples (Fig. 4g, h); *VvCAL* transcripts were only detected in WS and RWS cells (Fig. 4k). Among aquaporin genes, *VvPIP1;1* was one of the most highly expressed in embolism-inducing treatments (WS and PC), but it was also activated in RWS and RPC cells (Fig. 4i). *VvPIP1;2* expression was undetectable in all treatments (data not shown). *VvPIP2;1* was more expressed in PC and RPC treatments than in the other treatments (Fig. 4j); however, *VvPIP2;4N* transcripts were only observed in WS and mostly in RWS cells (Fig. 4l). Considering the members of the LEA family, which are typically involved in plant stress response, *VvDHN1a* was exclusively expressed in WS and PC cell samples, whereas *VvLEA14* was mainly activated in PC and in RPC cells and to a lesser extent in WS cells (Fig. 4m, n).

To verify the specificity of gene expression in VACs, quantitative real-time PCR (RT-qPCR) experiments were performed on the same target genes working on whole petiole samples. *VvGPT1* and *VvSUC27* expression followed the same patterns observed in VACs (Fig. 5a, b). *VvBAM3* was significantly activated upon WS treatment (as in VACs), but it was down-regulated in PC and RPC petioles, despite that these latter expression changes were not significant when compared with the IRR control (Fig. 5c). The two *LEA* genes were both strongly up-regulated upon WS treatment and their expression was still very high in RWS samples (Fig. 5d, e). Moreover, *VvLEA14* was

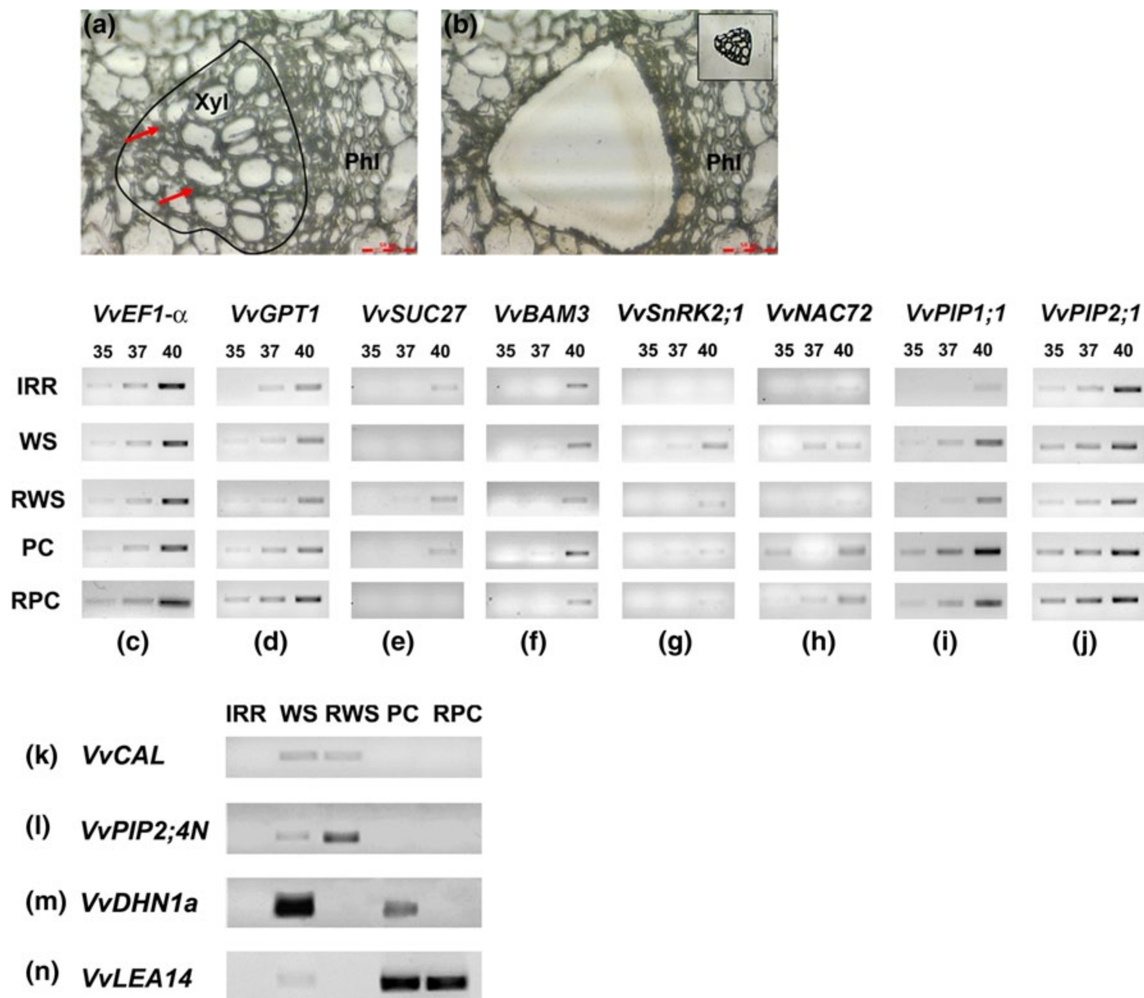


Fig. 4 Microdissection of vessel-associated cells around xylem vessels (target section area is indicated with a *black line*) before (**a**) and after (**b**) laser cutting. The *inset* shows collected cells; *red arrows* indicate vessel-associated cells. Pictures were taken using a ×40 objective lens; *scale bars* represent 50 µm. *Xyl* xylem cells, *Phl* phloem cells. **c**–**j** Semi-quantitative RT-PCR analyses on microdissected cells using the elongation factor gene (*VvEF1-α*) (**c**) as endogenous control. Numbers correspond to RT-PCR cycles. **k**–**n** RT-PCR on microdissected cells using specific primers for *VvCAL* (**k**), *VvPIP2;4 N* (**l**), *VvDHN1a* (**m**) and *VvLEA14* (**n**) genes. The size of amplified sequences is 100 bp. *IRR* irrigated control, *WS* water stress, *RWS* recovery from water stress, *PC* pressure collar stress, *RPC* recovery from pressure collar stress

significantly over-expressed in PC petioles, mirroring the pattern observed in VACs (Fig. 5d). No significant changes were observed for *VvDHN1a* transcripts in PC samples compared to the IRR control (Fig. 5e), while the same gene was activated in VACs upon this treatment.

In whole petiole, the expression of genes encoding components of signal transduction followed patterns similar to those observed in VACs: both *VvNAC72* and *VvSnRK2*;*1* levels increased in WS and RWS treatments (Fig. 5f, g), although the over-expression of *VvSnRK2;1* was significant only in WS samples (Fig. 5g). Interestingly, *VvNAC72* was also slightly activated in PC and RPC treatments (Fig. 5f), whereas *VvSnRK2*;*1* transcripts underwent a down-regulation in the same samples (Fig. 5g).

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

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

Finally, while the expression of *VvPIP2*;*1* followed the same pattern observed in VACs (it strongly increased

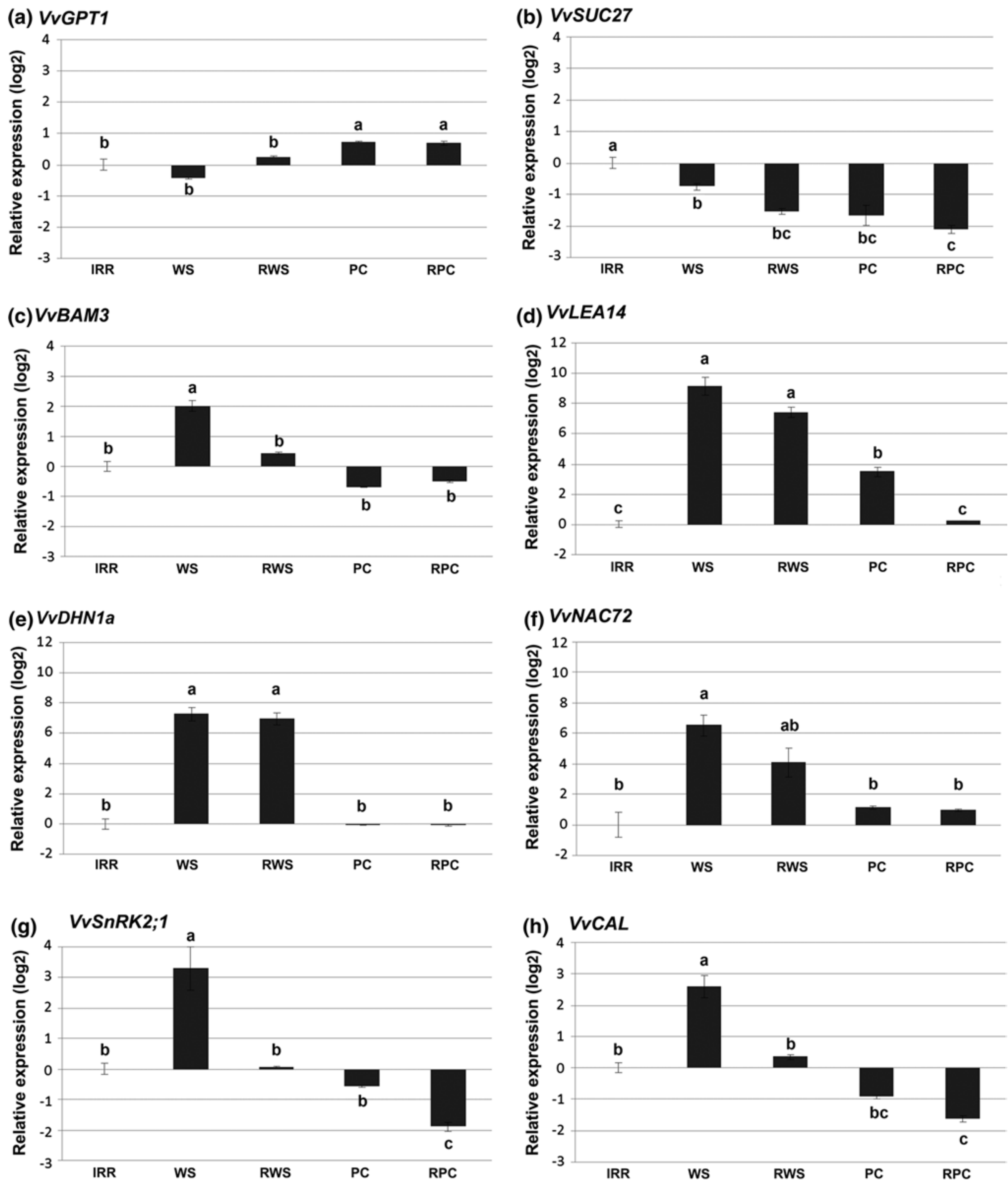
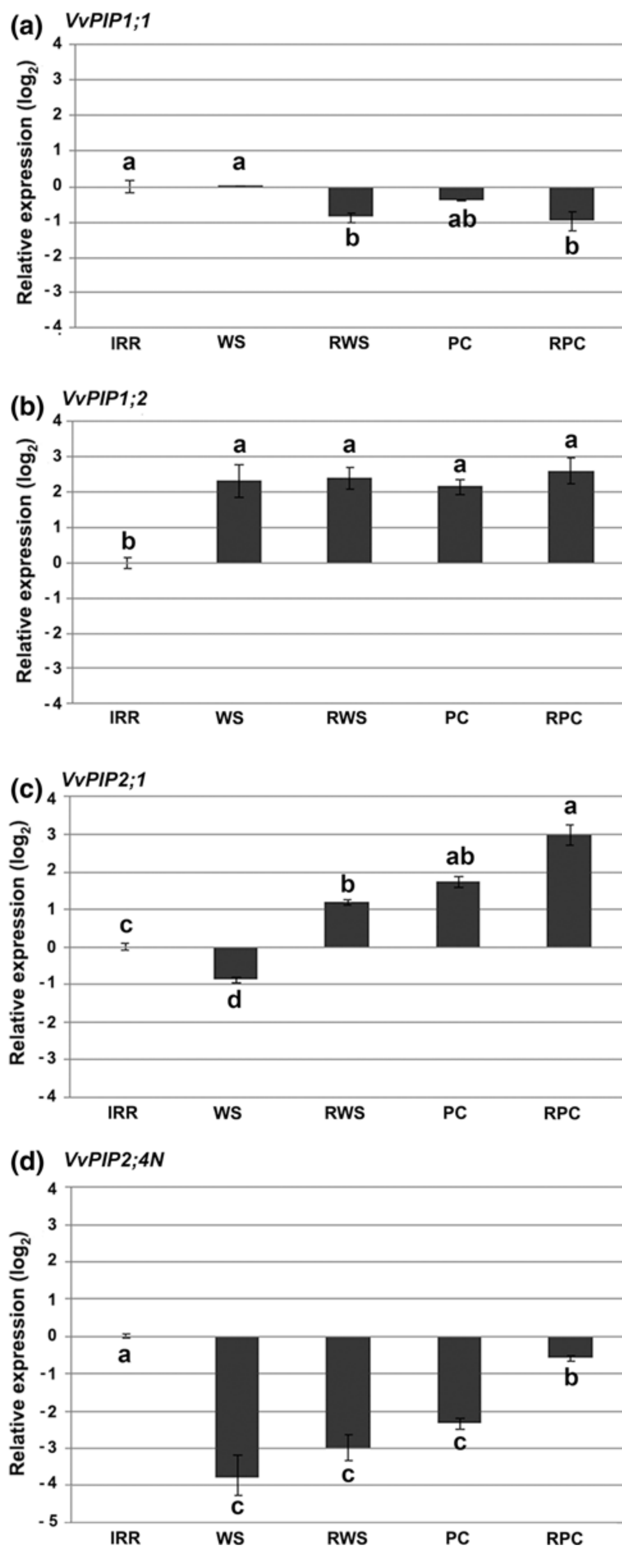


Fig. 5 Expression analysis of target genes in whole petioles: **a**–**h** RT-qPCR analyses on cv. Grenache petioles for *VvGPT1* (**a**), *VvSUC27* (**b**), *VvBAM3* (**c**), *VvLEA14* (**d**), *VvDHN1a* (**e**), *VvNAC72* (**f**), *VvSnRK2;1* (**g**) and *VvCAL* (**h**) transcripts. *Ubiquitin* (*VvUBI*)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



◂**Fig. 6** Expression analysis of target aquaporin genes in whole petioles: **a–d** RT-qPCR analyses on cv. Grenache petioles for *VvPIP1;1* (**a**), *VvPIP1;2* (**b**), *VvPIP2;1* (**c**) and *VvPIP2;4 N* (**d**) transcripts. *Ubiquitin* (*VvUBI*) and *Actin1* (*VvACT1*) were used as endogenous control genes for the normalisation procedure. *IRR* irrigated control, *WS* water stress, *RWS* recovery from water stress, *PC* pressure collar stress, *RPC* recovery from pressure collar stress. Different *lowercase letters* denote significant differences ($P < 0.05$) attested by using the Tukey's test; *bars* are standard errors of the mean ($n = 3$)

in PC and RPC), *VvPIP2;4N* transcriptional levels were significantly down-regulated in all treatments (Fig. 6c, d).

Discussion

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

It is well known that water stress (and subsequent rehydration) can induce xylem embolism formation and recovery. Nevertheless, this environmental condition triggers a wide array of molecular changes, which can mask those strictly related to embolism refilling processes. To control these masking effects, we employed, parallel to water stress and rehydration, the technique of stem pressurisation/depressurisation to induce embolism formation and repair with a limited incidence of other stress-induced processes. Both water stress and stem pressurisation require petiole excision to assess the degree of embolism, and this was reported to induce artefacts on *Acer rubrum* and *Fraxinus americana* (Wheeler et al. 2013). However, direct observations of embolism recovery obtained in the absence of petiole excision (Brodersen et al. 2010, 2013) suggest that refilling in grapevine is not affected by such artefacts (Sperry 2013).

In our experiment, stem pressurisation was obtained by applying a pressure collar to grape stems. Other authors have already used artificial tools to induce xylem cavitation in woody plants (e.g. Salleo et al. 1996; Mayr et al. 2006; Secchi and Zwieniecki 2011). These systems are particularly suited to increasing pressure gradients at air–water interfaces into the plant organs, thus inducing embolism formation. The method we set up avoids both air injection bores and wounding to create the air inlet. After about 5 h of PC treatment, PLC increased from 10 to 55 % in petioles; following depressurisation, PLC fully recovered within 3 h.

The PC treatment was not devoid of effects on water potential and leaf gas exchange, which decreased after pressurisation and recovered upon depressurisation. Nevertheless, the time courses of PLC, water potential and leaf gas exchange upon RPC and RWS were clearly different, since RPC recovery kinetics were more rapid. Depressurised twigs of laurel, previously submitted to a pressure collar treatment, showed faster and larger xylem refilling

than upon native embolism repair (Salleo et al. 1996). A slow recovery of hydraulic conductance and transpiration after rehydration of drought-exposed plants has been well documented in grapevine and linked to the persistence of high ABA concentration after rehydration (Lovisolo et al. 2008; Flexas et al. 2009; Zufferey et al. 2011; Perrone et al. 2012b). 'Grenache' is a drought-avoiding isohydric grape genotype, particularly suited to study drought responses, since it is able to tolerate long-term water stress conditions (Schultz 2003; Soar et al. 2004; Vandeleur et al. 2009) through ABA-mediated control of stomatal closure (Lovisolo et al. 2008, 2010). In this experiment, recovery in RPC plants was indeed associated with low leaf ABA concentration.

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

Expression changes of genes putatively involved in embolism recovery

Embolism recovery is an active process, which requires energy and metabolic activity. It takes place upon negative tensions in the xylem, and several mechanistic models have been proposed to explain it. All these models converge in considering the pivotal role of solutes, solute transporters and water transport facilitators (aquaporins) in VACs. Recently, two studies based on transcriptomic analysis of tissues undergoing embolism recovery have reported some genes linked to these processes, which undergo significant expression changes (Secchi et al. 2011; Perrone et al. 2012b). Nevertheless, these studies have focused on whole tissues, where molecular processes localised in VACs may not be detectable. To deepen the role of these genes, we have analysed the expression of some of these transcripts in VACs isolated by LMD.

The importance of regulation of carbohydrate metabolism and transport in VACs during the embolism recovery process has already been supported by physiological analyses (Salleo et al. 1996; Nardini et al. 2011; Secchi et al. 2013) and measurements of gene expression changes (Secchi et al. 2011; Perrone et al. 2012b). *VvBAM3* encodes a beta-amylase up-regulated by water stress (Perrone et al. 2012b) as its *Arabidopsis* orthologue (Fulton et al. 2008). *VvGPT1* is annotated as a plastidic glucose-6P symporter and is up-regulated upon embolism recovery (Perrone et al. 2012b). Its closest *Arabidopsis* homologue, the Glc6P/phosphate translocator1 (*At*GPT1), is localised in vascular bundle sheath cells (Niewiadomski et al. 2005), where it contributes to glucose-6-phosphate transport into plastids (Kunz et al. 2010). In grape petioles, *Vv*GPT1 could operate in the reverse direction, providing a supply of GLU-6P into the cytosol of VACs. *VvGPT1* and *VvBAM3* genes were both activated in VACs upon the embolism-inducing treatments applied (WS and PC). These data are in agreement with a picture of activated starch hydrolysis and GLU-6P export from plastids, which provide soluble sugars required to support the embolism recovery process. *Vv*SUC27 is an H^+-dependent sucrose transporter, whose expression is associated with sink organs in grape (Davies et al. 1999). In whole grape petioles, *VvSUC27* is down-regulated by all treatments inducing embolism formation, and it has previously been observed to be also down-regulated due to water stress (Perrone et al. 2012b). This suggests that, upon xylem embolism, the main provision of sugars to VACs derives from starch breakdown and not from phloem unloading. *VvSUC27* expression was almost absent in VACs, where phloem cells are not present. However, the regulatory changes involving these genes were not limited to VACs. Indeed, they were also detected in whole petioles, suggesting that most of the petiole cells collaborate with each other in the mobilisation of soluble sugars that drives embolism recovery.

The picture was quite different in the case of aquaporins, which are thought to facilitate water supply to the xylem, thus determining a successful refilling process (Kaldenhoff et al. 2008). This hypothesis requires that the activation of these channels takes place in the cells surrounding xylem vessels. Among the tested *PIP1* and *PIP2* genes, *VvPIP1;1*, *VvPIP2;1* and *VvPIP2;4N* were expressed in VACs of either embolising or recovering petioles, confirming a potential role for these proteins in embolism refilling. However, in the case of *VvPIP1;1* and *VvPIP2;4N* genes, these expression differences were not observed in whole petioles, both in this study and in a previous work by Perrone et al. (2012b), suggesting that their activation was strictly localised in VACs. The role of aquaporins in embolism refilling has been inferred from expression measurements performed in different plants, such as olive (Secchi et al. 2007), grapevine (Galmés et al. 2007), rice (Sakurai-Ishikawa et al. 2011), tobacco (Mahdieh et al. 2008) and

poplar (Secchi et al. 2011). Aquaporins could contribute to embolism refilling only indirectly by facilitating axial flow of water to the leaves and thus reducing the xylem tension gradient. Our expression results, obtained for the first time at the VAC level, strengthen the hypothesis that these aquaporins play a pivotal role in refilling xylem embolism.

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

Water stress and pressure collar responses to xylem embolism

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

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

We further measured the expression of three stress-responsive genes involved in signal transduction (*VvCAL, VvSnRK2;1, VvNAC72*). In detail, *VvCAL* is the grape orthologue of the *AtCLM24* (AT5G37770) gene, which encodes a Ca^{2+} binding protein in response to ABA stimulus, day length and salt stress (Delk et al. 2005). *VvSnRK2;1* encodes a protein kinase involved in ABA signal transduction, strongly up-regulated in grape leaves treated with exogenous ABA (Boneh et al. 2012). *VvNAC72* is the grape orthologue of *AtNAC72* (AT4G27410), whose expression is strictly controlled by ABA (Fujita et al. 2004); in grape petioles this gene is activated upon water stress (Perrone et al. 2012b). Our results indicate that *VvCAL* is only activated in WS and RWS VACs, and in WS whole petioles; *VvSnRK2;1* transcripts are more abundant upon WS both in VACs and whole petioles, although a slight up-regulation of this gene could be observed in VACs upon PC, RPC and RWS treatments. On the contrary, *VvNAC72* is more expressed in PC and RPC VACs.

Such differences between the two treatments were also observed for genes putatively related to embolism recovery. Expression of *VvPIP2;4N* and to a lesser extent of *VvBAM3* increased upon water stress. *VvPIP2;4N* is a root-specific grape aquaporin (Perrone et al. 2012a) and the localisation of its expression in VACs, depending on water stress, could explain the fact that this gene is not detected in whole petioles. In olive twigs, it has been shown that *OePIP2;1* aquaporin expression is activated when shoot hydraulic conductance recovers (Secchi et al. 2007), and generally there is an up-regulation of aquaporin genes when rehydration also occurs in grapevine leaves (Galmés et al. 2007) or petioles (Perrone et al. 2012b). In addition, a coupling of aquaporin activation with an increment in leaf transpiration has also been reported in rice roots, where transpiration demand triggers the up-regulation of PIPs localised both at the proximal end of the endodermis and on the cell surface around xylem (Sakurai-Ishikawa et al. 2011), and in drought-exposed/rehydrated tobacco roots (Mahdieh et al. 2008). An obvious candidate for gene activation exclusively under drought stress is a surge in ABA concentration, and correspondingly we found no ABA increase in PC and RPC-treated leaves. We have previously shown (Lovisolo et al. 2008; Perrone et al. 2012b) that, upon rehydration from water stress, grapevine leaves accumulate ABA at levels even higher than during the stress itself, and this could be instrumental to embolism recovery if contemporaneously VAC-specific aquaporins are activated as is the case of *Vv*PIP2;4N.

Other genes (*VvGPT1* and *VvPIP2;1*) are present only upon pressure collar pressurisation and depressurisation. These treatments thus trigger embolism-induced signals that are not induced in water-stressed plants, although embolism is also present in the latter. An explanation for this apparently contradictory result can be found in the different dynamics of embolism induction and recovery deriving from the two types of treatment. These dynamics are much faster in PC and RPC treatments. This means that, during PC and RPC treatments, a fast induction of embolism could elicit signals that are not present when a slow induction of embolism occurs, such as the case of water stress treatment. Secchi and Zwieniecki (2010), also using an artificial device to induce formation of xylem embolism in poplar, proposed some possible signals evoked during fast embolism induction, such as the accumulation of soluble sugars in the xylem or oxidative stress. However, in natural (and agricultural) conditions, xylem embolism almost invariably arises because of drought. The experimental use of devices, such as the pressure collar, which is applied to obtain embolism in the absence of water stress, could not

be representative of this condition, since the application of these tools seems to activate genes that are not expressed by water stress and following recovery.

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