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**Title:** Recovery from water stress affects grape leaf petiole transcriptome.

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Abstract

Fast and efficient recovery from water stress is a key determinant of plant adaptation to changing meteorological conditions modulating transpiration, i.e. air temperature and humidity. We analysed transcriptomic responses during rehydration after water stress in grapevine leaf petioles, where embolism formation and repair take commonly place, and where metabolic changes related to embolism recovery are expected to be particularly important.

We compared gene expression of recovering plants with irrigated controls, upon high and low transpiration conditions, using cDNA microarrays. In parallel, we assessed the daily dynamics of water relations, embolism formation and repair, and leaf abscisic acid concentration.

In recovering plants, the most affected gene categories were secondary metabolism, including genes linked to flavonoid biosynthesis; sugar metabolism and transport, including several aquaporin genes. The physiological dynamics of recovery were lower and the number of differentially expressed probes was much lower upon low transpiration than found in actively transpiring grapevines, suggesting the existence of a more intense metabolic reorganization upon high transpiration conditions and of a signal eliciting these responses. In plants recovering under high transpiration, abscisic acid concentrations significantly increased, and, in parallel, transcripts linked to abscisic acid metabolism and signalling (ABA-8'-hydroxylase, serine-threonine kinases, RD22 proteins) were upregulated; a trend that was not observed upon low transpiration.

Our results show that recovery from water stress elicits complex transcriptomic responses in grapevine. The increases observed in abscisic acid cellular levels could represent a signal triggering the activation of responses to rehydration after stress.

Keywords: Aquaporins, Abscisic acid, Drought, Embolism, Microarrays, Transpiration, Vitis.

Abbreviations: ABA (Abscisic acid), IRR (Irrigated), WS (Water stress), REC (Recovery), HCFM (Hydraulic Conductance Flow Meter), qRT-PCR (Quantitative Real - Time PCR).
**Introduction**

Drought is a common environmental stress for grapevines, which are traditionally grown in soils characterized by low water content even when irrigated. Indeed, a mild water stress is considered beneficial to improve the quality of grapes, especially of those that are further processed for winemaking (Chaves et al. 2007).

Rehydration induces the reversal of many effects of water stress, but the dynamics of these processes are diverse: increase of water potential may be rapid (hours or even minutes), while recovery of transpiration and photosynthesis may require days (Kirschbaum 1988; Lovisolo et al. 2008a; Flexas et al. 2009; Zufferey et al. 2011). Non-irrigated crops in temperate climates and irrigated crops under arid climates are subjected to continuous cycles of water stress and rewatering. It is thus crucial for plants to enhance the efficacy and speed of recovery from water stress, in order to maximise growth and production.

Rehydration from water stress has been studied at the molecular level in desiccation-tolerant plants (Vicre et al. 2004). In Arabidopsis, using a transcriptomic approach, a specific activation of genes was shown, implying that water stress recovery is not a simple reversal of stress (Oono et al. 2003). However, to our knowledge, no information is available about transcriptomic changes occurring upon rehydration in crop plants.

Among short-term plant adaptations to water stress, xylem cavitation is one of the most effective, in particular where negative pressures drive water across relatively long distances, as in water-stressed grapevines (Schultz and Matthews 1988; Choat et al. 2010). As for several other effects of water stress, xylem embolism decreases when droughted plants are rewatered. Embolism formation is controlled by biophysical (surface tension of the xylem sap) and anatomical (vessel diameter, pit membrane structure) factors. On the contrary, embolism recovery is not simply a biophysical reversal of embolism formation, but it is tightly controlled and regulated by plant metabolism, as shown by the fact that it can occur at negative water potentials (Holbrook and Zwieniecki 1999; Melcher et al. 2001; Brodersen et al. 2010) and that it can be impaired by treatments affecting metabolic activity (Bucci et al. 2003; Salleo et al. 2004; Lovisolo and Schubert 2006; Lovisolo et al. 2008b). Several studies have provided clues about possible metabolic signals and processes that could control xylem embolism recovery, following diverse
experimental approaches (Sakr et al. 2003; Salleo et al. 2004; Domec et al. 2006; Lovisolo and Schubert 2006; Lovisolo et al. 2008b; Secchi and Zwieniecki 2010, 2011). However, scarce molecular evidence is available to date about genes that could play a role in the embolism recovery process.

The goal of this study was to profile gene expression during rewatering of water-stressed plants. We used as experimental system the grapevine, which normally experiences important xylem embolism under water stress (Schultz and Matthews 1988; Lovisolo and Schubert 2006). We sampled leaf petioles, where the daily changes in embolism formation and repair have previously been shown to follow a trend similar to what observed in shoots, and where metabolic events related to embolism recovery are expected to be particularly important (Lovisolo et al. 2008a). Indeed, since in grape tissues, physiological responses determined by water stress and embolism recovery strictly occur in perixylematic parenchyma cells (Salleo et al. 2004), petioles, because of their intrinsic anatomic characteristics, are well suited to study molecular changes triggering cycles of embolism formation and repair.

The effects of rehydration on gene expression were assessed in plants grown under different transpiration regimes, which are expected to affect the rate of recovery processes (Holbrook and Zwieniecki 1999; Galle et al. 2009; Brodersen et al. 2010).

The study was performed on Vitis vinifera cv Grenache, a genotype particularly suited to investigate responses to drought, since, being a typical isohydric variety, it well tolerates long-lasting water stress conditions (Schultz 2003; Soar et al. 2004; Vandeleur et al. 2009).

**Materials and methods**

**Plant material**

Seventy-two, two-years-old plants of Vitis vinifera L. ‘Grenache’ grafted on Vitis riparia × Vitis berlandieri 420A (Vivai Cooperativi Rauscedo – San Giorgio della Richinvelda (PN), Italy), were grown in a partially-climate-controlled glasshouse under natural light and photoperiod conditions. Each plant grew in a 10 l container 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 (10 February) to the beginning of the experimental period, plants were irrigated twice every week to maintain water container capacity (Lovisolo and Schubert 1998). Lateral shoots were removed and plants had 25 leaves at the time of the first experiment, and 30 at the time of the second experiment.

Experimental set-up

Recovery from water stress was studied in two different experiments, where rehydration was applied on water stressed plants under different levels of atmospheric water demand. In the first experiment, water stress was applied on 36 plants in a period of low atmospheric evaporative water demand (start June; Experiment A). A second experiment was carried out on a different set of 36 plants under conditions of high atmospheric evaporative water demand (end July: experiment B). Maximum temperature and minimum relative humidity in the days of measurement and sampling were respectively 26°C and 60% in June, and 38°C and 24% in July.

In order to induce water stress, irrigation was withheld in each experiment on two thirds of the plants until leaf water potential had reached levels lower than -1.20 MPa. At the beginning of the first following day ("experimental day"), one half of the droughted plants was rehydrated (at 07.00 h) by watering pots to container capacity. Three treatments were thus obtained: irrigated (IRR), water stress (WS), and recovery from water stress (REC), each applied to 12 plants.

Starting one hour after rewatering, we followed for 12 hours the dynamics of leaf water potential, leaf gas exchange, embolism formation and repair, and ABA content in leaves. Measurements were done in both experiments at 2, 5, 8, and 11 h after rewatering (HAR: corresponding to 9.00; 12.00; 15.00; 18.00 h), at each time point on a different set of three plants per treatment. Petioles for molecular analyses were also sampled on the same time points.

Measurements of leaf gas exchange, leaf water potential, extent of xylem embolism, and ABA analysis
Leaf transpiration rate, stomatal conductance and vapour pressure deficit (VPD) were recorded using an infra-red gas analyser ADC-LCPro+ system (The Analytical Development Company Ltd) on one leaf per plant inserted in the central region of the shoot (nodes 6 to 18 from the shoot base). Leaf water potential ($\Psi_{\text{leaf}}$) was thereafter measured on two different transpiring leaves per plant inserted in the central region of the shoot, using a Scholander-type pressure chamber (Soil Moisture Equipment Corp.).

Xylem embolism was assessed immediately after and on the same plants where $\Psi_{\text{leaf}}$ had been measured, on leaf petioles, following the protocol described by Lovisolo et al. (2008a) and using a Hydraulic Conductance Flow Meter (HCFM-XP of Dynamax Inc.) (Tyree et al. 1995). Briefly, one further leaf per plant was chosen in the central region of the shoot and petioles were excised underwater 1 cm from the nodes by bending the shoot and submerging the petiole into a water container. Petioles were connected underwater to the HCFM, and a few seconds after starting the measurement the leaf was cut 1 cm above the petiole insertion. The extent of xylem embolism was determined by comparing the initial hydraulic conductivity ($K_{\text{hi}}$) of petioles with their maximum final hydraulic conductivity ($K_{\text{hf}}$), recorded after removing air emboli by means of transient water flushing, and was expressed as percent loss of conductivity $\text{PLC} (%) = 100 \times (K_{\text{hf}} - K_{\text{hi}})/(K_{\text{hf}})$. The extent of embolism recovery was estimated as difference between PLC of irrigated plants and of recovering plants ($\text{PLC_{REC-Irr}}$). Conductivity measurements were performed within 1 h from start of sampling.

Leaf ABA content was measured on the same leaf used for conductivity measurements, following the method described in (Lovisolo et al. 2008a).

RNA extraction, microarray construction and hybridization

For REC and IRR treatments, RNA was extracted from petioles collected at the same time points where physiological measurements were made, whereas in experiment B we extracted RNA only from petioles collected at 8 HAR. Extraction of RNA from petioles of WS plants was done in both experiments on samples collected at 8 HAR. Three petioles (about 2 g in total), one respectively from the apical, central and basal parts of each plant, were detached, frozen in liquid nitrogen, and stored at -80°C. After grinding petioles in liquid nitrogen, total RNA was extracted following
the protocol described by (Carra et al. 2007). RNA integrity and quantity were checked with a 2100 Bioanalyzer (Agilent), using the RNA LabChip® assay. Once known the exact RNA yields, samples were treated with the proper concentration of DNase I, RNase-free (Fermentas: 50 U µL⁻¹) in according with the manufacturer's instructions, to successfully avoid any risk of DNA contamination. Moreover, to further enhance purity of the DNase-treated RNA samples, before microarray analysis, a phenol : chloroform : isoamyl alcohol 25 : 24 : 1 (by vol.) purification step, followed by precipitation in sodium acetate and absolute ethanol, was also performed, and RNA quality and concentrations were checked again.

Microarray experiments were performed using the grape AROS V1.0 platform (http://www.operon.com), which is based on a set of 14562 70-mer oligonucleotide probes, each specific for one of the ESTs and EST contigs (named TCs, Tentative Consensuses) contained in the Vitis vinifera Gene Index (VvGI, release 5) (http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=grape). The glass chips were prepared at CRIBI (University of Padova, Italy), as described by Trainotti et al. (2006). The probes, harbouring a 5’ amino linker, were spotted onto glass chips (MICROMAX Glass Slides Superchip™ I, Perkin Elmer) in 48 subgrids (4 columns x 12 rows) and each probe was spotted twice in the same chip. Twelve probes carrying the role of positive controls were distributed in each subgrid. Reference spots, obtained from a mix of all positive control oligos, were deposited at constant positions in each subgrid to have information about the spatial variability of the hybridization signal. Twelve randomly generated negative controls were included in each subgrid.

Fluorescently labelled cDNA was synthesized, purified, and used for hybridization of the microarrays, as described in Supplemental Materials and methods.

Microarray experimental design and data analysis

The effects of water stress were analysed by comparing gene expression in WS and IRR leaf petioles collected at 8 HAR: to this aim, WS and IRR tissue samples from both experiments were respectively pooled before RNA extraction. The effects of rehydration were analysed by comparing gene expression of REC and IRR leaf petioles collected at the same time after rewatering. In Exp. A, a time course of transcriptional regulation under recovery was obtained by carrying out
comparisons at all sampling times (2; 5; 8 and 11 HAR), while in Exp. B expression changes were
analysed only at 8 HAR. Each comparison was based on two biological replicates, where the
green-fluorescent dye (Cy3) was coupled to either the WS or REC cDNAs and the red-fluorescent
dye (Cy5) to the control (IRR) cDNA, and a technical replicate obtained by swapping the dye-
labelling on the same cDNA samples. The total number of slides used was thus 12 for Exp. A (4
time points x 1 comparison x 3 replicates), 3 for Exp. B (1 time point x 1 comparison x 3
replicates) and 3 for WS versus IRR comparison.

Microarray slides were scanned as described by Trainotti et al. (2006), using a two-channel
confocal microarray scanner (ScanArray1 Lite, Perkin-Elmer), setting the laser power emission
between 77% and 82% and the Photomultiplier tube (PMT) between 73% and 75%. Microarray
images were analyzed using TIGR Spotfinder v.2.2.4 (www.tm4.org/spotfinder.html), applying the
Otsu algorithm, and visually rechecked. The resulting raw data were normalized by applying a
Locally Weighted Linear Regression (LOWESS) using the Microarray Data Analysis (TIGR MIDAS,

Normalized expression data were statistically analysed by one-class unpaired Significance
Analysis of Microarray (SAM) (Tusher et al. 2001) using the Multi Experiment Viewer (TIGR MeV,
v.4.0, www.tm4.org/mev) software, and applying a 66% cut-off filter prior to the analysis. Clones
with significant changes in expression were identified at values giving a 10% false discovery rate
(FDR). Only those probes resulting significant at the SAM analysis and that contemporarily
reached the expression ratio threshold of log2=1 were retained for further analyses. Probe
annotations were updated by query of the respective TC nucleotide sequence at the VvGI database
(release 5) against the UniProt/TrEMBL protein database, using the blastx algorithm with a
minimum significance value (e-value) of 1e-10, using the software Blast2GO v.1.7.2
(www.blasto2go.de). Probes were further annotated following the Gene Ontology (GO) functional
classification (biological process) (Ashburner et al. 2000) (www.geneontology.org), using the GO
mapping step of Blast2GO. GO terms of differentially expressed probes were thus grouped into
functional classes using the AgBase GOslimViewer software with Plants filter
(http://agbase.msstate.edu/cgi-bin/tools/goslimviewer_select.pl). Functional classes were
arbitrarily pooled into higher-order functional categories (Suppl. Table S1). The homogeneity of
frequencies of hits falling in the functional categories was finally tested by a $\chi^2$ test.
Quantitative PCR analysis of gene expression

First-strand cDNA was synthesized in triplicate starting from 10 µg of total RNA, extracted as described above, using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) and according to the manufacturers’ instructions.

Gene-specific primers (reported in Suppl. Table S2) for quantitative Real Time-PCR (qRT-PCR) assay were designed using the Primer Express® software (v3.0, Applied Biosystems) on the complete *Vitis* cDNA sequences, retrieved from the Genoscope 12X database (http://www.genoscope.cns.fr), corresponding to the TC sequences of the target probes. Only in the case of aquaporin genes, qRT-PCR primers were designed on the heterogeneous 3’ untranslated region (UTR), in order to discriminate among the different isoforms of the target aquaporin genes. Reactions were carried out in the StepOnePlus™ Real-Time PCR System (Applied Biosystems) and the SYBR Green method (Power SYBR® Green PCR Master Mix, Applied Biosystems) was chosen for quantifying the amplification results, as previously described (Hugueney et al. 2009). Gene expression was quantified after normalization to the housekeeping genes ubiquitin (*VvUBI, VvGI release 7 TC117219*) and actin1 (*VvACT1, VvGGI release 7 TC134791*). Results were calculated as expression ratios (relative quantity, RQ) between either WS or REC samples and IRR controls.

**Results**

Physiological responses to drought and rehydration

During both periods of water deprivation (ten days in Exp. A and seven days in Exp. B), leaf water potential ($\Psi_{\text{leaf}}$) progressively decreased to about -1.4 MPa, with no significant differences between the two experiments. Nevertheless, $\Psi_{\text{leaf}}$ recovered at different rates in the two experiments: in Exp. A, $\Psi_{\text{leaf}}$ reached -0.4 MPa within five hours from rewatering, but it took two hours only to reach the same level in Exp. B (Fig. 1).
Diurnal vapour pressure deficit (VPD) was about 12 mbar bar\(^{-1}\) with slight variations along the experimental day in Exp. A, while in Exp. B VPD increased above 30 mbar bar\(^{-1}\) in the morning, and remained higher than in Exp. A during the rest of the day (Fig. 2a).

Stomatal conductance \((g_s)\) increased up to 15.00 h (8 HAR) in Exp. A to maximum values around 0.35 mol H\(_2\)O m\(^{-2}\) s\(^{-1}\), while in Exp. B maximum \(g_s\) of irrigated plants was about one third than in Exp. A. In WS plants, \(g_s\) values were very low (less than 0.05 mol H\(_2\)O m\(^{-2}\) s\(^{-1}\)) in both experiments. In REC plants, \(g_s\) was similar to that of WS plants at the beginning of the day, and then it increased reaching about 50% that of IRR controls (Fig. 2b).

Leaf transpiration (E) was high in Exp. A, but it was even higher in Exp. B, due to the high VPD and notwithstanding the low stomatal conductance. In IRR plants, E increased up to 15.00 h (8 HAR) in Exp. A to about 4 mmol m\(^{-2}\) s\(^{-1}\), while in Exp. B maximum E was about 6 mmol m\(^{-2}\) s\(^{-1}\). In WS plants, E was low and similar in both experimental periods. The transpiration rate (E) of REC plants followed a pattern intermediate between IRR and WS plants, and was higher in Exp. B than in Exp. A (Fig. 2c). Net photosynthesis (A), which was not affected by VPD, showed a pattern similar as observed for stomatal conductance (Fig. 2d), resulting in lower values of instantaneous water use efficiency (WUE\(i = A/E\)) in Exp. B than in Exp. A. Sub-stomatal CO\(_2\) concentration was correspondingly lower in REC plants than in IRR controls in Exp. A. On the contrary, it was higher in REC than in IRR plants in Exp. B, suggesting a non-stomatal regulation of photosynthesis (Fig. 2e).

Water stress induced extensive xylem embolism, with PLC values between 60 and 70% (Fig. 3). Rehydration induced a steady decrease of PLC in both experiments, albeit with different intensities: in the 11 h after rewatering, PLC decreased to 26% in Exp. A and to 60% in Exp. B. The time course of petiole PLC confirmed that grapevines are submitted to diurnal cycles of embolism formation and repair. These cycles were evident in IRR plants, and occurred (even if not significantly) also in REC plants of Exp. B. On the contrary, almost no daily PLC variation was observed in WS plants in both experiments (Fig. 3).

In Exp. A, under low transpiration rate, ABA concentration in leaves remained constant at a basal level of about 2000 pmol g\(^{-1}\) DW in IRR controls. In REC plants, ABA concentration showed no significant differences in comparison to IRR controls in the first and last parts of the day (3, 5 and
11 HAR), but it was lower than in IRR controls at 8 HAR. On the contrary, in Exp. B, ABA concentration significantly increased in REC plants as compared to IRR controls (Fig. 4).

Patterns of gene regulation under water stress and rehydration

In the microarray comparison between WS and IRR plants, the number of probes spotted on the chip that showed significant hybridization differences after SAM analysis (FDR 10%), and that also satisfied the expression ratio threshold chosen (log$_2$>1), was 358, where 207 probes were upregulated and 151 downregulated.

On the base of the same threshold parameters, in plants recovering after rehydration, in conditions of low transpiration (Exp. A), 144 probes showed significant expression differences in at least one of the four comparisons. In particular, 21 probes were upregulated and 123 downregulated. Most differences in gene expression were observed just after rewatering (2 HAR), where probes showing a significant difference were 118 at 2 HAR, 11 at 5 HAR, 25 at 8 HAR, and 30 at 11 HAR. In last, in plants recovering under high transpiration (Exp. B), 407 probes showed significant hybridization differences and, among them, 179 underwent transcriptional activation, whereas 228 were downregulated.

Functional grouping of GO hits assigned most differentially expressed genes to the categories response to stress, cell growth and morphogenesis, reproduction, cell cycle and homeostasis, and protein and aminoacid metabolism. Significant differences from equal distribution at the $\chi^2$ test were observed, in particular in Exp B, where the incidence of hits falling in the photosynthesis and energy, carbohydrate metabolism, and transport was significantly higher, while hits in the cell wall and morphogenesis and reproduction, cell cycle and homeostasis were significantly less (Table 1).

The functional categories were also differently affected by up- and downregulation in the different treatments and experiments as indicated by the ratio between the percentage of upregulated probes and of downregulated probes (or vice-versa) for each category (referred in the text respectively as U/D and D/U). The comparison of expression levels in Exp. A (all data points pooled together) showed positive effects of the rehydration treatment in the cell growth and
morphogenesis (U/D = 4.3), lipid metabolism (4.3), and carbohydrate metabolism (2.1) categories, and a negative effect on the response to stress (D/U = 4.06), secondary metabolism (3.57) and nucleic acid metabolism categories (2.8). In Exp. B, positive effects of the rehydration treatment were observed on the secondary metabolism (U/D = 4.26) and carbohydrate metabolism (2.00), and negative effects on the photosynthesis and energy (D/U = 9.05) categories (Fig. 5).

Among the 144 probes differentially regulated in REC plants compared to IRR controls in Exp. A, 32 were also differentially affected by water stress (13 upregulated and 19 downregulated). Among the 407 probes differentially regulated in REC plants compared to IRR controls in Exp. B, 93 were also significantly affected by water stress (47 upregulated and 46 downregulated), while 22 were also differentially regulated by rehydration in Exp. A (2 upregulated and 17 downregulated) (Fig. 6).

Expression changes upon recovery

Within each functional category, differential regulation exclusively induced by rehydration (REC) often followed different patterns in the two experiments (Suppl. Tables S4 and S6).

Photosynthesis and energy

In conditions of low transpiration, at the first time point (2 HAR), three transcripts involved in respiration, photorespiration and fermentation (cytochrome c reductase: TC62094; glycolate oxidase: TC52919; alcohol dehydrogenase: TC63091) were downregulated. A RUBISCO binding protein, thought to act as chaperone for RUBISCO assembly (TC52052), was activated at 5 HAR, while other photosynthesis-related genes, such as a chlorophyll binding protein (TC56895), were downregulated at later time points. Nevertheless, comparing the two experiments, the repression of energy metabolism was more evident upon high transpiration conditions, where several genes, as chlorophyll-binding proteins (TC54828, TC56028, TC55189, TC57521, CB341233, TC65556), subunits of photosystems I and II (TC54542, TC52528, TC61693), and oxygen-evolving enhancer proteins associated to photosystem II (TC54765, TC53930), were significantly downregulated.
One transcript involved in fermentation (alcohol dehydrogenase, TC63091) was downregulated in both conditions.

**Carbohydrate metabolism**

Under low transpiration a pattern of general downregulation was observed for this category, at the beginning of the time-course (2 HAR), where a sucrose synthase (TC59920) and a glycosyltransferase (TC53455) were the most downregulated transcripts. Upon high transpiration, the expression of enzymes involved in galactose metabolism (e.g. GDP-mannose 3,5 epimerase: TC58885; TC55380), and of a fructokinase (TC57339) was reduced, while a gene encoding an alpha-amylase (TC67671) was significantly activated.

**Nucleic acid metabolism, transcription and translation**

In condition of low transpiration, this category was dominated by the downregulation of zinc-finger transcription factors (BM437359, TC62891), and by the activation of a DEAD-box RNA-binding protein (TC65154). On the contrary, upon high transpiration, we did not observe significant expression changes in transcription, with the only exception of a MYBA1 transcription factor (TC64719), a key regulator of flavonoid biosynthesis, which was upregulated in parallel with the many transcripts of this pathway (see below).

**Secondary metabolism**

In Exp. A, several genes involved in flavonoid biosynthesis (3-phosphoshikimate 1-carboxyvinyltransferase-EPSP synthase: TC69881; chalcone synthase: TC67855; phenylalanine-ammonia-lyase: TC61248 and TC66528; flavanone-3-hydroxylase: TC67181; leucoanthocyanidin dioxygenase: TC69652,) were downregulated at 2 and at 5 HAR. Two GSTs (TC61062 and CB342131) were also downregulated at 2 HAR, in agreement with a possible function in flavonoid transport into vacuoles.

On the contrary, in Exp. B most of the genes involved in phenylpropanoid and flavonoid biosynthesis were coordinately activated by rehydration, including (in biosynthetic order): 4-coumarate-CoA ligase (TC57438), chalcone synthase (TC67409), chalcone isomerase (TC55034), dihydroflavonol 4-reductase (TC53437; TC51699), flavonol synthase (TC57653), UFGT (TC63201), and anthocyanidin reductase (TC55505).
Transport

Upon low transpiration conditions, this category was not particularly affected: only three transcripts were differentially expressed at 2 and 5 HAR. On the contrary, in Exp. B, genes linked to transport were strongly activated, in particular several members of the PIP-type aquaporin family, including three PIP1 (TC51776, TC60619, TC55780) and two PIP2 (TC58240, TC69190). Also one transporter, a TT12-like protein (TC62162, TC69833), possibly related to flavonoid accumulation was found among upregulated genes in this category.

Response to endogenous stimuli and signal transduction

In Exp. A, this functional class was overall downregulated. Reduced expression was observed for two Ca- and calmodulin-binding proteins (CB009010, CF208534), a RING-finger protein (TC53052), possibly involved in protein/protein interactions, a RLK-type S/T protein kinase (BM437868), member of a large family of plant genes that are involved in responses to biotic and abiotic stress (Lehti-Shiu et al. 2009), and a LEA protein (BE846412), reflecting a downscaling of osmotic adaptations. A downregulation of Ca$^{2+}$ and calmodulin-binding proteins (TC53352; TC55454) was observed also in Exp. B; however an inactivation of protein kinases and receptor-like kinases (TC66054, TC63041, TC53071) was specifically observed only upon high transpiration conditions. In these conditions, ethylene and IAA pathways were also inactivated, through the downregulation of an ethylene receptor (TC52433), of ACC oxidase (TC56709), and of AUX/IAA proteins (TC51731, TC64438, TC60981). Always in Exp. B, we observed the activation of the major gene responsible for ABA catabolism (ABA 8’-hydroxylase, TC67543) (Nambara and Marion-Poll 2005).

Cell growth and morphogenesis

Upon low transpiration, in most gene categories early downregulation of genes activated by water stress was the rule, whereas a different pattern was observed for probes involved in cell growth, such as XET (TC63269), and BRU1, a brassinosteroid-induced protein showing extensive homology with XET (Zurek and Clouse 1994), which were upregulated later in the day. This was confirmed by analysis of the probes affected by both water stress and recovery, among whom those involved in this category (e.g. two XETs, CF212592 and CB346454) followed a consistent late reversal of the effects of water stress (Suppl. Table S6). On the contrary, upon high transpiration, this category was affected in an opposite way, as several probes involved in cell
enlargement, such as arabinogalactan proteins (TC65487; TC67718), a COBRA-like protein involved in definition of cell polarity (TC68223), an expansin (TC68519) and two cellulose synthases (TC56114; TC59569) were downregulated and only one probe, encoding phytosulfokine (TC66424) and involved in cell proliferation, was upregulated.

Many of the results above reported were confirmed also through the analysis of those probes that were contemporarily regulated by WS and by REC treatments. For instance, in Exp. A, the two XET genes (CF212592 and CB346454) upregulated by REC were instead downregulated by WS (Suppl. Table S5).

In Exp. B, several probes involved in photosynthesis (e.g. RUBISCO, CB809963 and TC70003) were downregulated both by REC and by WS, whereas two probes involved in secondary metabolism (4-coumarate-CoA ligase TC62308 and leucoanthocyanidin reductase TC61972) were upregulated by both treatments (Suppl. Table S7).

Validation of microarray data by qRT-PCR

Expression changes of a group of transcripts, selected among those significantly differentially expressed in microarrays, were quantified by Quantitative Real-Time PCR (qRT-PCR) in petiole extracts used for the comparisons WS/IRR (11 genes) and REC/IRR of Exp. B sampled at 8 HAR (14 genes). For the majority of target genes (8/11 genes for WS/IRR and 14/14 genes for REC/IRR), the pattern of up- or downregulation, observed in microarray results, was confirmed by qRT-PCR analysis, but in the case of three transcripts in the WS/IRR comparison (sucrose transporter, potassium transporter, glucose-6-P translocator), the patterns diverged (Suppl. Table S3). These variations probably depend on the fact that i) some regions of the Vitis genome are still not definitely described (Zenoni et al. 2011), and ii) we amplified cDNA from the Grenache cultivar, while the sequenced genome originates from the PN40024 clone, and cultivar-specific polymorphisms are commonly observed in grapevine. For these reasons, the high specificity of qRT-PCR in detecting target transcripts could have induced the formation of amplicons different than expected.
**Discussion**

Physiological and molecular processes of recovery from water stress are affected by transpiration intensity.

As judged from water potential and gas exchange measurements, in both experiments, plants reached the same levels of water stress, comparable to those normally observed in *Vitis vinifera cv 'Grenache'* (Lovisolo et al. 2010). However, recovery from water stress took place under different temperature and evaporative conditions, resulting in higher VPDs and lower stomatal conductances in Exp. B than in Exp A. Higher transpirations in Exp. B occurred thanks to the higher VPDs, despite vines in that experiment experienced low levels of stomatal conductance even in irrigated conditions, as predicted in near-isohydric grape cultivars downregulating stomatal opening when VPD increases (Soar et al. 2006).

The dynamics of leaf water potential recovery were different in the two experiments, and under low transpiration leaf water potential following rehydration increased more slowly than in Exp. B. This observation implies that, upon high transpiration, water flew rapidly along the non-embolized petiole xylem and parenchyma cells, notwithstanding the fact that at rewatering the non-embolized conductivity was lower than upon low transpiration (18% vs 37% in Exp. A, measured as 100% minus PLC). This induced a rapid increase of leaf water potential, while, upon low transpiration, leaves drew less water, thus recovering water potential at a slower pace.

Embolism recovery also followed different dynamics in the two experiments. Following water stress, plants showed a high level of petiole xylem embolism (PLC) in both experiments. In the 11 hours after rehydration, PLC decreased in the REC plants to a similar extent (about 20% more than irrigated controls) in both conditions of transpiration, as evidenced by the REC-IRR line in Fig. 3. However, recovery was faster under high transpiration, where it was completed by about two thirds at 5 HAR; while the same level of recovery in Exp. A was reached after 8 HAR.

Are the different dynamics of water potential and embolism recovery causally connected? A first hypothesis can be based on embolism recovery being essentially controlled by xylem water tension, which we estimate to be equal to leaf water potential as soil water potential was kept constant in the two experiments. In this hypothesis, upon rewatering water would flow along the
non embolized and the recovering vessels to the leaves, thus increasing leaf water potential and releasing tension, which would in turn allow physical re-absorption of the cavitations. This hypothesis is satisfied by the fact that embolism recovery was faster upon high transpiration, where stress recovery was faster. However, recovery from water stress has been shown to consist not only in a passive hydraulic process, but also to be based on active mechanisms supported by metabolic processes (Holbrook and Zwieniecki 1999; Oono et al. 2003; Salleo et al. 2004). Thus a second, additional hypothesis to explain the different dynamics of recovery in the two experiments is that high transpiration may evoke a non-hydraulic signal able to induce regulatory and metabolic processes. The obvious candidate for such role is ABA, which is transported in the grapevine xylem (Downton et al. 1988), and whose delivery to the leaf could be modulated by leaf transpiration rate. In a previous paper (Lovisolo et al. 2008a), we reported a surge of ABA concentration in petioles recovering from water stress under high transpiration, with levels even higher than in petioles of water stressed plants, and we argued that this was due to fast transport of ABA to recovering leaves by the transpiration stream. In this study, we confirm that, under high transpiration, leaf ABA concentration increases in REC leaves compared with IRR leaves, but furthermore we show that, under low transpiration conditions (Exp. A), this ABA surge is absent and a slight increase of ABA concentration can be measured only in the last part of the day. The different patterns of ABA accumulation in recovering plants under different levels of transpiration were in agreement with corresponding changes of stomatal conductivity, confirming reports of stomatal closure under recovery from water stress at high transpiration (Lovisolo et al. 2008a; Flexas et al. 2009). These results open the hypothesis that, in addition to its well-known role in controlling stomatal conductance in grape (Soar et al. 2004), ABA could also trigger active processes of recovery from water stress in presence of high transpiration.

Transcriptome modifications induced by rehydration may underlie water potential and embolism recovery.

Under both transpiration regimes, rewatering induced changes in gene expression profiles. In Exp A, where we profiled expression changes at four time-points along the day following rewatering,
the highest number of differentially expressed probes was observed at the first sampling point (2
HAR). At this stage many genes, involved in particular in carbohydrate, nucleic acid and protein
metabolism, secondary metabolism, responses to stress, and signal transduction underwent
negative regulation, suggesting a general inactivation of stress-induced metabolisms and, in
parallel, of the related signalling processes. Later on (11 HAR), genes involved in growth and
morphogenesis were activated. Thus, in grape petioles, the recovery process under low
transpiration conditions broadly follows the model described in Arabidopsis by Oono et al. (2003),
with an early reversal of stress followed by an activation of growth and development.

The physiological differences observed during recovery under different transpiration regimes were
mirrored by quantitative and qualitative differences of gene expression. Comparing samples
collected at the same time after rewatering (8 HAR), in Exp. A only 25 probes showed a significant
alteration in transcript concentration, whilst in Exp. B 407 probes were differentially expressed.
Even though the genes differentially expressed by water stress are substracted from those
differentially regulated by rewatering in Exp. B (i.e. if expression changes of these genes are
supposed to be only a later effect of water stress) the results are respectively 12 and 314,
indicating that a much more intense gene expression reorganization takes place upon rewatering
upon high than low transpiration conditions.

A few trends of gene regulation, observed under recovery in conditions of low transpiration, were
confirmed under high transpiration. This was the case of the general downregulation of genes
involved in photosynthesis. This suggests that, during recovery from water stress, photosynthesis
is limited in petioles, in agreement with the reduction of the efficiency of the photosynthetic
system, which was described in grape and tobacco leaves recovering from water stress in
comparable conditions (Flexas et al. 2009; Galle et al. 2009).

However, under high transpiration new and unexpected patterns of gene regulation emerged. One
was the case of the general inactivation of genes involved in cell growth and morphogenesis (as
arabinogalactans and expansins). This observation, in agreement with the downregulation of
photosynthesis, suggests a general arrest of growth processes in leaf petioles recovering under
high transpiration. Downregulation of growth and photosynthesis could allow a redirection of
metabolism towards active processes of recovery, which could be at the base of the faster recovery
observed in these conditions.
Secondary metabolism was inhibited under recovery in Exp. A, while it was generally activated in Exp. B; in particular, genes representing most of the phenylpropanoid and flavonoid biosynthetic pathways were activated. In addition, a transcription factor (VvmybA2), which specifically upregulates anthocyanin biosynthesis in grape (Kobayashi et al. 2002; Fournier-Level et al. 2009), and two glutathione-S-transferases (GSTs) were also activated. Besides other metabolic roles, evidences for GST involvement in vacuolar transport of flavonoids have already been reported in grape (Ageorges et al. 2006).

Moreover, since secondary metabolism also provides substrates for the polymerization of lignin and condensed tannins, which are present in the xylem of young grape stems (Jordao et al. 2001), the activation of this gene category could be necessary if new xylem vessels are developing during recovery from water stress, in order to sustain an important water demand from the transpiring leaves.

The Transport functional group was generally activated by rehydration, together with several amino acid permeases, which could be involved in a reorganization of protein biosynthesis, and a few MATE transporters. A role for this protein family in anthocyanin delivery into vacuoles has recently been demonstrated by Gomez et al. (2011). However, the main feature emerging from the expression data was the activation of plasma membrane aquaporins (PIPs). The members of this subfamily of proteins are normally expressed in different grape leaf organs, including petioles (Baiges et al. 2001), where they facilitate transcellular water transport. The role of petiole aquaporins in the recovery process may be double. On one side, aquaporins can contribute to accelerate axial cell-to-cell movement of water through the cortical parenchyma of leaf petioles, favouring recovery of water potential: this hypothesis, however, must take into account that, at the time of sampling (8 HAR), the water potential had completely recovered since 2 hours at least.

Another possibility is that they contribute to refill embolized vessels by facilitating radial water flow from parenchyma cells to xylem vessels, in agreement with the fact that embolism recovery was still active at the moment of sampling. Several studies have proposed the involvement of aquaporins in embolism recovery, where they would facilitate the transfer of water from parenchyma cells to embolized xylem vessels (Martre et al. 2002; Sakr et al. 2003; Kaldenhoff et al. 2008; Secchi and Zwieniecki 2010). Although our observation cannot prove such a role, it is interesting to note that the activation of aquaporins was gene-specific: only the water-transporting
VvPIP2;1 was upregulated, together with three PIP1 genes, which do not transport water when expressed in *Xenopus* oocytes (Vandeleur et al. 2009) and are though to have regulatory functions (Kaldenhoff and Fischer 2006).

The accumulation of soluble sugars in the embolized xylem vessels has been proposed to serve as an osmotic driving force for attracting water (through aquaporins) within the embolized vessels that are at negative pressures (Salleo et al. 1996), or as a signal of vessel embolism (Secchi and Zwieniecki 2011). In recovering shoots, we observed the activation of an alpha-amylase, which could be involved in hydrolysing starch, as proposed in poplar by Secchi and Zwieniecki (2011). On the contrary, no activation of sugar transporters was observed, indicating that either this process is less important in grape than in poplar, or that these transporters are post-transcriptionally regulated.

An ABA signal may induce molecular responses to rehydration in grapevine

Gene expression changes in recovering plants under conditions of low and high transpiration were often contrasting, implying that different signals are originated in these two conditions. One of these signals can be ABA, as we observed no differences in ABA concentration between water-stressed and recovering plants under low transpiration, while, under high transpiration, we measured a surge of ABA in recovering plants. In a previous paper (Lovisolo et al. 2008a), we speculated that the ABA surge in leaves recovering upon high transpiration depends on passive transport from the roots, due to the rise in water flux following rehydration, and accordingly, in the present work, we do not see activation of ABA biosynthetic genes in recovering petioles, but only of ABA-metabolizing genes, such as ABA-8′-hydroxylase. The ABA signal activates wide transcriptional responses in Arabidopsis (Seki et al. 2002; Matsui et al. 2008). When applied to grape berries, ABA activates expression of flavonoid biosynthetic genes and pathogen response proteins (Jeong et al. 2004; Giribaldi et al. 2010). In recovering petioles under high transpiration, the increase in ABA concentration was matched by well-known transcriptional effects of ABA, such as the activation of RD22 proteins and of ABA-catabolyzing enzymes, such as ABA-8′-hydroxylase. Other probes upregulated in this experiment represent genes known to be activated by ABA, such as TC54154, a calmodulin-like protein (Delk et al. 2005), and TC56494, a serine-
threonine kinase (Kobayashi et al. 2004, 2005). An increase of endogenous ABA upon recovery may represent an after-effect of water stress, controlling, besides stomatal closure, other checkpoints of water transport throughout the plant during rehydration, thus affecting the speed and dynamics of recovery from water stress. One such role of ABA could be in the control of aquaporin expression. It has been shown that ABA can upregulate PIP-type aquaporins in roots of Arabidopsis (Jang et al. 2007), maize (Parent et al. 2009) and tobacco (Mahdieh and Mostajeran 2009), and in leaves of bean (Montalvo-Hernandez et al. 2008). The results of other studies, where high transpiration upregulates shoot aquaporins but where ABA was not measured, could also be explained by such this mechanism. Transpiration demand triggers an increase in PIP2 expression in rice roots at the cell surface around the xylem (Sakurai-Ishikawa et al. 2011); shade-grown poplars experiencing a sudden increase in light, enhancing transpiration, exhibit increased transcript abundance of 15 aquaporin genes (Almeida-Rodriguez et al. 2011). The transcriptional effect of ABA on aquaporins, however, may be complex: an attenuation of transpiration is required for the enhancement of membrane permeability and the upregulation of aquaporins in the plasma membrane of maize protoplasts (Morillon and Chrispeels 2001). In Arabidopsis leaves, xylem-born ABA represses water mesophyll conductance, possibly through inactivation of aquaporins (Shatil-Cohen et al. 2011). ABA could thus selectively act on specific members of the aquaporin family, in order to alleviate the effects of water stress. Further research, involving e.g enhanced xylem delivery of exogenous ABA to embolized xylem vessels, will be needed to test this hypothesis.

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Captions to figures

**Fig. 1** Leaf water potential ($\Psi_{\text{leaf}}$) measured on Grenache plants irrigated (IRR), subjected to water stress (WS), or rewatered (REC) in the two experiments (A and B). Arrow shows time of rehydration. Bars are standard errors of the means ($n=6$).

**Fig. 2** Vapour pressure deficit (VPD) (a), stomatal conductance ($g_s$) (b), leaf transpiration (E) (c), net photosynthesis A (d), and substomatal CO$_2$ concentration (Ci) (e) measured on Grenache plants irrigated (IRR), subjected to water stress (WS), or rewatered (REC) in the two experiments (A and B). Arrow shows time of rehydration. Bars are standard errors of the means ($n=3$).

**Fig. 3** Percent loss of conductivity (PLC) changes measured on leaf petioles of Grenache plants irrigated (IRR), subjected to water stress (WS), or rewatered (REC) in Exp. A (a) and in Exp. B (b). Arrow shows time of rehydration. Bars are standard errors of the means ($n=3$). REC-IRR values (circles) have been calculated by subtracting to PLC values recorded in recovering plants the values measured under irrigated conditions in order to draw a virtual trend line showing the net incidence of embolism formation (increase of PLC) or repair (decrease of PLC) during plant recovery.

**Fig. 4** Time course of foliar ABA contents measured in leaves of Grenache plants irrigated (IRR) or rewatered (REC) in Exp. A (a) and in Exp B (b). Arrow shows time of rehydration. Bars are standard errors of the means ($n=3$).

**Fig. 5** Percent incidence of GO hits of up- and downregulated probes showing significant expression differences and reaching the log$_2$$>$1 threshold of expression ratio in the REC plants relative to IRR plants in all the time points of Exp. A (a), and in the REC plants relative to the IRR of Exp. B (b).

**Fig. 6** Venn diagrams showing the number of probes whose expression was up- or downregulated (reaching the log$_2$$>$1 threshold of expression ratios) by water stress (WS) or rehydration (REC).
relative to IRR controls at all the time points in Exp. A (above left), and in Exp. B (above right),
and by rehydration in the two experiments (REC A and REC B) (below).
Fig. 1
Fig. 4

Foliar ABA (pmol g⁻¹ DW)

IRR a

REC a

IRR b

REC b
Fig. 5

a

photosynthesis and energy

carbohydrate metabolism

nucleic acids metabolism

protein and aminoacid metabolism

lipid metabolism

secondary metabolism

response to endogenous stimuli and signal...

response to stress

cell growth and morphogenesis

transport

reproduction, cell cycle and homeostasis

b

photosynthesis and energy

carbohydrate metabolism

nucleic acids metabolism

protein and aminoacid metabolism

lipid metabolism

secondary metabolism

response to endogenous stimuli and signal...

response to stress

cell growth and morphogenesis

transport

reproduction, cell cycle and homeostasis

up

down

upregulated

downregulated
Fig. 6

EXP A

REC UP
9

WS UP
194

WS DOWN
132

REC DOWN
103

EXP B

REC UP
128

WS UP
160

WS DOWN
105

REC DOWN
186

Fig. 6