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

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# UNIVERSITÀ DEGLI STUDI DI TORINO

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14

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25

26

27 **Abstract**

28

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

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

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

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

50

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

52

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

## 55 **Introduction**

56

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

61 Rehydration induces the reversal of many effects of water stress, but the dynamics of these  
62 processes are diverse: increase of water potential may be rapid (hours or even minutes), while  
63 recovery of transpiration and photosynthesis may require days (Kirschbaum 1988; Lovisolo et al.  
64 2008a; Flexas et al. 2009; Zufferey et al. 2011). Non-irrigated crops in temperate climates and  
65 irrigated crops under arid climates are subjected to continuous cycles of water stress and  
66 rewatering. It is thus crucial for plants to enhance the efficacy and speed of recovery from water  
67 stress, in order to maximise growth and production.

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

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

85 experimental approaches (Sakr et al. 2003; Salleo et al. 2004; Domec et al. 2006; Lovisolo and  
86 Schubert 2006; Lovisolo et al. 2008b; Secchi and Zwieniecki 2010, 2011). However, scarce  
87 molecular evidence is available to date about genes that could play a role in the embolism  
88 recovery process.

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

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

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

105

106

## 107 **Materials and methods**

108

109 Plant material

110

111 Seventy-two, two-years-old plants of *Vitis vinifera* L. 'Grenache' grafted on *Vitis riparia* × *Vitis*  
112 *berlandieri* 420A (Vivai Cooperativi Rauscedo – San Giorgio della Richinvelda (PN), Italy), were  
113 grown in a partially-climate-controlled glasshouse under natural light and photoperiod conditions.

114 Each plant grew in a 10 l container filled with a substrate composed of a sandy-loam soil (pH 7.0;

115 available P 7.9 mg kg<sup>-1</sup>; organic matter 1.37%; cation exchange capacity 4.58 meq 100 g<sup>-1</sup>/expanded clay/peat mixture (2:1:1 by weight). From budbreak (10 February) to the beginning of  
116 the experimental period, plants were irrigated twice every week to maintain water container  
117 capacity (Lovisollo and Schubert 1998). Lateral shoots were removed and plants had 25 leaves at  
118 the time of the first experiment, and 30 at the time of the second experiment.

120

121 Experimental set-up

122

123 Recovery from water stress was studied in two different experiments, where rehydration was  
124 applied on water stressed plants under different levels of atmospheric water demand. In the first  
125 experiment, water stress was applied on 36 plants in a period of low atmospheric evaporative  
126 water demand (start June; Experiment A). A second experiment was carried out on a different set  
127 of 36 plants under conditions of high atmospheric evaporative water demand (end July:  
128 experiment B). Maximum temperature and minimum relative humidity in the days of  
129 measurement and sampling were respectively 26°C and 60% in June, and 38°C and 24% in July.  
130 In order to induce water stress, irrigation was withheld in each experiment on two thirds of the  
131 plants until leaf water potential had reached levels lower than -1.20 MPa. At the beginning of the  
132 first following day (“experimental day”), one half of the droughted plants was rehydrated (at 07.00  
133 h) by watering pots to container capacity. Three treatments were thus obtained: irrigated (IRR),  
134 water stress (WS), and recovery from water stress (REC), each applied to 12 plants.

135 Starting one hour after rewatering, we followed for 12 hours the dynamics of leaf water potential,  
136 leaf gas exchange, embolism formation and repair, and ABA content in leaves. Measurements  
137 were done in both experiments at 2, 5, 8, and 11 h after rewatering (HAR: corresponding to 9.00;  
138 12.00; 15.00; 18.00 h), at each time point on a different set of three plants per treatment. Petioles  
139 for molecular analyses were also sampled on the same time points.

140

141 Measurements of leaf gas exchange, leaf water potential, extent of xylem embolism, and ABA  
142 analysis

143

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

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

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

165

166 RNA extraction, microarray construction and hybridization

167

168 For REC and IRR treatments, RNA was extracted from petioles collected at the same time points  
169 where physiological measurements were made, whereas in experiment B we extracted RNA only  
170 from petioles collected at 8 HAR. Extraction of RNA from petioles of WS plants was done in both  
171 experiments on samples collected at 8 HAR. Three petioles (about 2 g in total), one respectively  
172 from the apical, central and basal parts of each plant, were detached, frozen in liquid nitrogen,  
173 and stored at -80°C. After grinding petioles in liquid nitrogen, total RNA was extracted following

174 the protocol described by (Carra et al. 2007). RNA integrity and quantity were checked with a  
175 2100 Bioanalyzer (Agilent), using the RNA LabChip® assay. Once known the exact RNA yields,  
176 samples were treated with the proper concentration of DNase I, RNase-free (Fermentas: 50 U  $\mu\text{L}^{-1}$ )  
177 in according with the manufacturer's instructions, to successfully avoid any risk of DNA  
178 contamination. Moreover, to further enhance purity of the DNase-treated RNA samples, before  
179 microarray analysis, a phenol : chloroform : isoamyl alcohol 25 : 24 : 1 (by vol.) purification step,  
180 followed by precipitation in sodium acetate and absolute ethanol, was also performed, and RNA  
181 quality and concentrations were checked again.

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

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

196

197 Microarray experimental design and data analysis

198

199 The effects of water stress were analysed by comparing gene expression in WS and IRR leaf  
200 petioles collected at 8 HAR: to this aim, WS and IRR tissue samples from both experiments were  
201 respectively pooled before RNA extraction. The effects of rehydration were analysed by comparing  
202 gene expression of REC and IRR leaf petioles collected at the same time after rewatering. In Exp.  
203 A, a time course of transcriptional regulation under recovery was obtained by carrying out

204 comparisons at all sampling times (2; 5; 8 and 11 HAR), while in Exp. B expression changes were  
205 analysed only at 8 HAR. Each comparison was based on two biological replicates, where the  
206 green-fluorescent dye (Cy3) was coupled to either the WS or REC cDNAs and the red-fluorescent  
207 dye (Cy5) to the control (IRR) cDNA, and a technical replicate obtained by swapping the dye-  
208 labelling on the same cDNA samples. The total number of slides used was thus 12 for Exp. A (4  
209 time points x 1 comparison x 3 replicates), 3 for Exp. B (1 time point x 1 comparison x 3  
210 replicates) and 3 for WS versus IRR comparison.

211 Microarray slides were scanned as described by Trainotti et al. (2006), using a two-channel  
212 confocal microarray scanner (ScanArray1 Lite, Perkin-Elmer), setting the laser power emission  
213 between 77% and 82% and the Photomultiplier tube (PMT) between 73% and 75%. Microarray  
214 images were analyzed using TIGR Spotfinder v.2.2.4 ([www.tm4.org/spotfinder.html](http://www.tm4.org/spotfinder.html)), applying the  
215 Otsu algorithm, and visually rechecked. The resulting raw data were normalized by applying a  
216 Locally Weighted Linear Regression (LOWESS) using the Microarray Data Analysis (TIGR MIDAS,  
217 v.2.19, [www.tm4.org/midas.html](http://www.tm4.org/midas.html)) software.

218 Normalized expression data were statistically analysed by one-class unpaired Significance  
219 Analysis of Microarray (SAM) (Tusher et al. 2001) using the Multi Experiment Viewer (TIGR MeV,  
220 v.4.0, [www.tm4.org/mev](http://www.tm4.org/mev)) software, and applying a 66% cut-off filter prior to the analysis. Clones  
221 with significant changes in expression were identified at values giving a 10% false discovery rate  
222 (FDR). Only those probes resulting significant at the SAM analysis and that contemporarily  
223 reached the expression ratio threshold of  $\log_2=1$  were retained for further analyses. Probe  
224 annotations were updated by query of the respective TC nucleotide sequence at the VvGI database  
225 (release 5) against the UniProt/TrEMBL protein database, using the blastx algorithm with a  
226 minimum significance value (e-value) of  $1e^{-10}$ , using the software Blast2GO v.1.7.2  
227 ([www.blasto2go.de](http://www.blasto2go.de)). Probes were further annotated following the Gene Ontology (GO) functional  
228 classification (biological process) (Ashburner et al. 2000) ([www.geneontology.org](http://www.geneontology.org)), using the GO  
229 mapping step of Blast2GO. GO terms of differentially expressed probes were thus grouped into  
230 functional classes using the AgBase GOslimViewer software with Plants filter  
231 ([http://agbase.msstate.edu/cgi-bin/tools/goslimviewer\\_select.pl](http://agbase.msstate.edu/cgi-bin/tools/goslimviewer_select.pl)). Functional classes were  
232 arbitrarily pooled into higher-order functional categories (Suppl. Table S1). The homogeneity of  
233 frequencies of hits falling in the functional categories was finally tested by a  $\chi^2$  test.

234 Quantitative PCR analysis of gene expression

235

236 First-strand cDNA was synthesized in triplicate starting from 10  $\mu$ g of total RNA, extracted as  
237 described above, using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems)  
238 and according to the manufacturers' instructions.

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

252

253

## 254 **Results**

255

256 Physiological responses to drought and rehydration

257

258 During both periods of water deprivation (ten days in Exp. A and seven days in Exp. B), leaf water  
259 potential ( $\Psi_{\text{leaf}}$ ) progressively decreased to about -1.4 MPa, with no significant differences between  
260 the two experiments. Nevertheless,  $\Psi_{\text{leaf}}$  recovered at different rates in the two experiments: in  
261 Exp. A,  $\Psi_{\text{leaf}}$  reached -0.4 MPa within five hours from rewatering, but it took two hours only to  
262 reach the same level in Exp. B (Fig. 1).

263 Diurnal vapour pressure deficit (VPD) was about 12 mbar bar<sup>-1</sup> with slight variations along the  
264 experimental day in Exp. A, while in Exp. B VPD increased above 30 mbar bar<sup>-1</sup> in the morning,  
265 and remained higher than in Exp. A during the rest of the day (Fig. 2a).

266 Stomatal conductance ( $g_s$ ) increased up to 15.00 h (8 HAR) in Exp. A to maximum values around  
267 0.35 mol H<sub>2</sub>O m<sup>-2</sup> s<sup>-1</sup>, while in Exp. B maximum  $g_s$  of irrigated plants was about one third than in  
268 Exp. A. In WS plants,  $g_s$  values were very low (less than 0.05 mol H<sub>2</sub>O m<sup>-2</sup> s<sup>-1</sup>) in both  
269 experiments. In REC plants,  $g_s$  was similar to that of WS plants at the beginning of the day, and  
270 then it increased reaching about 50% that of IRR controls (Fig. 2b).

271 Leaf transpiration (E) was high in Exp. A, but it was even higher in Exp. B, due to the high VPD  
272 and notwithstanding the low stomatal conductance. In IRR plants, E increased up to 15.00 h (8  
273 HAR) in Exp. A to about 4 mmol m<sup>-2</sup> s<sup>-1</sup>, while in Exp. B maximum E was about 6 mmol m<sup>-2</sup> s<sup>-1</sup>. In  
274 WS plants, E was low and similar in both experimental periods. The transpiration rate (E) of REC  
275 plants followed a pattern intermediate between IRR and WS plants, and was higher in Exp. B than  
276 in Exp. A (Fig. 2c). Net photosynthesis (A), which was not affected by VPD, showed a pattern  
277 similar as observed for stomatal conductance (Fig. 2d), resulting in lower values of instantaneous  
278 water use efficiency ( $WUE_i = A/E$ ) in Exp. B than in Exp. A. Sub-stomatal CO<sub>2</sub> concentration was  
279 correspondingly lower in REC plants than in IRR controls in Exp. A. On the contrary, it was  
280 higher in REC than in IRR plants in Exp. B, suggesting a non-stomatal regulation of  
281 photosynthesis (Fig. 2e).

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

289 In Exp. A, under low transpiration rate, ABA concentration in leaves remained constant at a basal  
290 level of about 2000 pmol g<sup>-1</sup> DW in IRR controls. In REC plants, ABA concentration showed no  
291 significant differences in comparison to IRR controls in the first and last parts of the day (3, 5 and

292 11 HAR), but it was lower than in IRR controls at 8 HAR. On the contrary, in Exp. B, ABA  
293 concentration significantly increased in REC plants as compared to IRR controls (Fig. 4).

294

295

296 Patterns of gene regulation under water stress and rehydration

297

298 In the microarray comparison between WS and IRR plants, the number of probes spotted on the  
299 chip that showed significant hybridization differences after SAM analysis (FDR 10%), and that  
300 also satisfied the expression ratio threshold chosen ( $\log_2 > 1$ ), was 358, where 207 probes were  
301 upregulated and 151 downregulated.

302 On the base of the same threshold parameters, in plants recovering after rehydration, in  
303 conditions of low transpiration (Exp. A), 144 probes showed significant expression differences in  
304 at least one of the four comparisons. In particular, 21 probes were upregulated and 123  
305 downregulated. Most differences in gene expression were observed just after rewatering (2 HAR),  
306 where probes showing a significant difference were 118 at 2 HAR, 11 at 5 HAR, 25 at 8 HAR, and  
307 30 at 11 HAR. In last, in plants recovering under high transpiration (Exp. B), 407 probes showed  
308 significant hybridization differences and, among them, 179 underwent transcriptional activation,  
309 whereas 228 were downregulated.

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

317 The functional categories were also differently affected by up- and downregulation in the different  
318 treatments and experiments as indicated by the ratio between the percentage of upregulated  
319 probes and of downregulated probes (or vice-versa) for each category (referred in the text  
320 respectively as U/D and D/U). The comparison of expression levels in Exp. A (all data points  
321 pooled together) showed positive effects of the rehydration treatment in the cell growth and

322 morphogenesis (U/D = 4.3), lipid metabolism (4.3), and carbohydrate metabolism (2.1) categories,  
323 and a negative effect on the response to stress (D/U = 4.06), secondary metabolism (3.57) and  
324 nucleic acid metabolism categories (2.8). In Exp. B, positive effects of the rehydration treatment  
325 were observed on the secondary metabolism (U/D = 4.26) and carbohydrate metabolism (2.00),  
326 and negative effects on the photosynthesis and energy (D/U = 9.05) categories (Fig. 5).  
327 Among the 144 probes differentially regulated in REC plants compared to IRR controls in Exp. A,  
328 32 were also differentially affected by water stress (13 upregulated and 19 downregulated). Among  
329 the 407 probes differentially regulated in REC plants compared to IRR controls in Exp. B, 93 were  
330 also significantly affected by water stress (47 upregulated and 46 downregulated), while 22 were  
331 also differentially regulated by rehydration in Exp. A (2 upregulated and 17 downregulated) (Fig.  
332 6).

333

334

335 Expression changes upon recovery

336

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

339

340 Photosynthesis and energy

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

351 One transcript involved in fermentation (alcohol dehydrogenase, TC63091) was downregulated in  
352 both conditions.

### 353 Carbohydrate metabolism

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

### 360 Nucleic acid metabolism, transcription and translation

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

### 367 Secondary metabolism

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

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

379

380

381 Transport

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

388 Response to endogenous stimuli and signal transduction

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

402 Cell growth and morphogenesis

403 Upon low transpiration, in most gene categories early downregulation of genes activated by water  
404 stress was the rule, whereas a different pattern was observed for probes involved in cell growth,  
405 such as XET (TC63269), and BRU1, a brassinosteroid-induced protein showing extensive  
406 homology with XET (Zurek and Clouse 1994), which were upregulated later in the day. This was  
407 confirmed by analysis of the probes affected by both water stress and recovery, among whom  
408 those involved in this category (e.g. two XETs, CF212592 and CB346454) followed a consistent  
409 late reversal of the effects of water stress (Suppl. Table S6). On the contrary, upon high  
410 transpiration, this category was affected in an opposite way, as several probes involved in cell

411 enlargement, such as arabinogalactan proteins (TC65487; TC67718), a COBRA-like protein  
412 involved in definition of cell polarity (TC68223), an expansin (TC68519) and two cellulose  
413 synthases (TC56114; TC59569) were downregulated and only one probe, encoding phytosulfokine  
414 (TC66424) and involved in cell proliferation, was upregulated.

415

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

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

424

425 Validation of microarray data by qRT-PCR

426

427 Expression changes of a group of transcripts, selected among those significantly differentially  
428 expressed in microarrays, were quantified by Quantitative Real-Time PCR (qRT-PCR) in petiole  
429 extracts used for the comparisons WS/IRR (11 genes) and REC/IRR of Exp. B sampled at 8 HAR  
430 (14 genes). For the majority of target genes (8/11 genes for WS/IRR and 14/14 genes for  
431 REC/IRR), the pattern of up- or downregulation, observed in microarray results, was confirmed by  
432 qRT-PCR analysis, but in the case of three transcripts in the WS/IRR comparison (sucrose  
433 transporter, potassium transporter, glucose-6-P translocator), the patterns diverged (Suppl. Table  
434 S3). These variations probably depend on the fact that i) some regions of the Vitis genome are still  
435 not definitely described (Zenoni et al. 2011), and ii) we amplified cDNA from the Grenache  
436 cultivar, while the sequenced genome originates from the PN40024 clone, and cultivar-specific  
437 polymorphisms are commonly observed in grapevine. For these reasons, the high specificity of  
438 qRT-PCR in detecting target transcripts could have induced the formation of amplicons different  
439 than expected.

440

441 **Discussion**

442

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

445

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

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

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

467 Are the different dynamics of water potential end embolism recovery causally connected? A first  
468 hypothesis can be based on embolism recovery being essentially controlled by xylem water  
469 tension, which we estimate to be equal to leaf water potential as soil water potential was kept  
470 constant in the two experiments. In this hypothesis, upon rewatering water would flow along the

471 non embolized and the recovering vessels to the leaves, thus increasing leaf water potential and  
472 releasing tension, which would in turn allow physical re-absorption of the cavitations. This  
473 hypothesis is satisfied by the fact that embolism recovery was faster upon high transpiration,  
474 where stress recovery was faster.

475 However, recovery from water stress has been shown to consist not only in a passive hydraulic  
476 process, but also to be based on active mechanisms supported by metabolic processes (Holbrook  
477 and Zwieniecki 1999; Oono et al. 2003; Salleo et al. 2004). Thus a second, additional hypothesis  
478 to explain the different dynamics of recovery in the two experiments is that high transpiration  
479 may evoke a non-hydraulic signal able to induce regulatory and metabolic processes. The obvious  
480 candidate for such role is ABA, which is transported in the grapevine xylem (Downton et al. 1988),  
481 and whose delivery to the leaf could be modulated by leaf transpiration rate. In a previous paper  
482 (Lovisol et al. 2008a), we reported a surge of ABA concentration in petioles recovering from water  
483 stress under high transpiration, with levels even higher than in petioles of water stressed plants,  
484 and we argued that this was due to fast transport of ABA to recovering leaves by the transpiration  
485 stream. In this study, we confirm that, under high transpiration, leaf ABA concentration increases  
486 in REC leaves compared with IRR leaves, but furthermore we show that, under low transpiration  
487 conditions (Exp. A), this ABA surge is absent and a slight increase of ABA concentration can be  
488 measured only in the last part of the day. The different patterns of ABA accumulation in  
489 recovering plants under different levels of transpiration were in agreement with corresponding  
490 changes of stomatal conductivity, confirming reports of stomatal closure under recovery from  
491 water stress at high transpiration (Lovisol et al. 2008a; Flexas et al. 2009). These results open  
492 the hypothesis that, in addition to its well-known role in controlling stomatal conductance in  
493 grape (Soar et al. 2004), ABA could also trigger active processes of recovery from water stress in  
494 presence of high transpiration.

495  
496 Transcriptome modifications induced by rehydration may underlie water potential and embolism  
497 recovery

498  
499 Under both transpiration regimes, rewatering induced changes in gene expression profiles. In Exp  
500 A, where we profiled expression changes at four time-points along the day following rewatering,

501 the highest number of differentially expressed probes was observed at the first sampling point (2  
502 HAR). At this stage many genes, involved in particular in carbohydrate, nucleic acid and protein  
503 metabolism, secondary metabolism, responses to stress, and signal transduction underwent  
504 negative regulation, suggesting a general inactivation of stress-induced metabolisms and, in  
505 parallel, of the related signalling processes. Later on (11 HAR), genes involved in growth and  
506 morphogenesis were activated. Thus, in grape petioles, the recovery process under low  
507 transpiration conditions broadly follows the model described in Arabidopsis by Oono et al. (2003),  
508 with an early reversal of stress followed by an activation of growth and development.

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

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

524 However, under high transpiration new and unexpected patterns of gene regulation emerged. One  
525 was the case of the general inactivation of genes involved in cell growth and morphogenesis (as  
526 arabinogalactans and expansins). This observation, in agreement with the downregulation of  
527 photosynthesis, suggests a general arrest of growth processes in leaf petioles recovering under  
528 high transpiration. Downregulation of growth and photosynthesis could allow a redirection of  
529 metabolism towards active processes of recovery, which could be at the base of the faster recovery  
530 observed in these conditions.

531 Secondary metabolism was inhibited under recovery in Exp. A, while it was generally activated in  
532 Exp. B; in particular, genes representing most of the phenylpropanoid and flavonoid biosynthetic  
533 pathways were activated. In addition, a transcription factor (VvmybA2), which specifically  
534 upregulates anthocyanidin biosynthesis in grape (Kobayashi et al. 2002; Fournier-Level et al.  
535 2009), and two glutathione-S-transferases (GSTs) were also activated. Besides other metabolic  
536 roles, evidences for GST involvement in vacuolar transport of flavonoids have already been  
537 reported in grape (Ageorges et al. 2006).

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

543 The Transport functional group was generally activated by rehydration, together with several  
544 amino acid permeases, which could be involved in a reorganization of protein biosynthesis, and a  
545 few MATE transporters. A role for this protein family in anthocyanin delivery into vacuoles has  
546 recently been demonstrated by Gomez et al. (2011). However, the main feature emerging from the  
547 expression data was the activation of plasma membrane aquaporins (PIPs). The members of this  
548 subfamily of proteins are normally expressed in different grape leaf organs, including petioles  
549 (Baiges et al. 2001), where they facilitate transcellular water transport. The role of petiole  
550 aquaporins in the recovery process may be double. On one side, aquaporins can contribute to  
551 accelerate axial cell-to-cell movement of water through the cortical parenchyma of leaf petioles,  
552 favouring recovery of water potential: this hypothesis, however, must take into account that, at  
553 the time of sampling (8 HAR), the water potential had completely recovered since 2 hours at least.  
554 Another possibility is that they contribute to refill embolized vessels by facilitating radial water  
555 flow from parenchyma cells to xylem vessels, in agreement with the fact that embolism recovery  
556 was still active at the moment of sampling. Several studies have proposed the involvement of  
557 aquaporins in embolism recovery, where they would facilitate the transfer of water from  
558 parenchyma cells to embolized xylem vessels (Martre et al. 2002; Sakr et al. 2003; Kaldenhoff et  
559 al. 2008; Secchi and Zwieniecki 2010). Although our observation cannot prove such a role, it is  
560 interesting to note that the activation of aquaporins was gene-specific: only the water-transporting

561 VvPIP2;1 was upregulated, together with three PIP1 genes, which do not transport water when  
562 expressed in *Xenopus* oocytes (Vandeleur et al. 2009) and are thought to have regulatory functions  
563 (Kaldenhoff and Fischer 2006).

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

572

573 An ABA signal may induce molecular responses to rehydration in grapevine

574

575 Gene expression changes in recovering plants under conditions of low and high transpiration were  
576 often contrasting, implying that different signals are originated in these two conditions. One of  
577 these signals can be ABA, as we observed no differences in ABA concentration between water-  
578 stressed and recovering plants under low transpiration, while, under high transpiration, we  
579 measured a surge of ABA in recovering plants. In a previous paper (Lovisolò et al. 2008a), we  
580 speculated that the ABA surge in leaves recovering upon high transpiration depends on passive  
581 transport from the roots, due to the rise in water flux following rehydration, and accordingly, in  
582 the present work, we do not see activation of ABA biosynthetic genes in recovering petioles, but  
583 only of ABA-metabolizing genes, such as ABA-8'-hydroxylase. The ABA signal activates wide  
584 transcriptional responses in *Arabidopsis* (Seki et al. 2002; Matsui et al. 2008). When applied to  
585 grape berries, ABA activates expression of flavonoid biosynthetic genes and pathogen response  
586 proteins (Jeong et al. 2004; Giribaldi et al. 2010). In recovering petioles under high transpiration,  
587 the increase in ABA concentration was matched by well-known transcriptional effects of ABA,  
588 such as the activation of RD22 proteins and of ABA-catabolizing enzymes, such as ABA-8'-  
589 hydroxylase. Other probes upregulated in this experiment represent genes known to be activated  
590 by ABA, such as TC54154, a calmodulin-like protein (Delk et al. 2005), and TC56494, a serine-

591 threonine kinase (Kobayashi et al. 2004, 2005). An increase of endogenous ABA upon recovery  
592 may represent an after-effect of water stress, controlling, besides stomatal closure, other  
593 checkpoints of water transport throughout the plant during rehydration, thus affecting the speed  
594 and dynamics of recovery from water stress. One such role of ABA could be in the control of  
595 aquaporin expression. It has been shown that ABA can upregulate PIP-type aquaporins in roots of  
596 Arabidopsis (Jang et al. 2007), maize (Parent et al. 2009) and tobacco (Mahdieh and Mostajeran  
597 2009), and in leaves of bean (Montalvo-Hernandez et al. 2008). The results of other studies, where  
598 high transpiration upregulates shoot aquaporins but where ABA was not measured, could also be  
599 explained by such this mechanism. Transpiration demand triggers an increase in PIP2 expression  
600 in rice roots at the cell surface around the xylem (Sakurai-Ishikawa et al. 2011); shade-grown  
601 poplars experiencing a sudden increase in light, enhancing transpiration, exhibit increased  
602 transcript abundance of 15 aquaporin genes (Almeida-Rodriguez et al. 2011). The transcriptional  
603 effect of ABA on aquaporins, however, may be complex: an attenuation of transpiration is required  
604 for the enhancement of membrane permeability and the upregulation of aquaporins in the plasma  
605 membrane of maize protoplasts (Morillon and Chrispeels 2001). In Arabidopsis leaves, xylem-  
606 borne ABA represses water mesophyll conductance, possibly through inactivation of aquaporins  
607 (Shatil-Cohen et al. 2011). ABA could thus selectively act on specific members of the aquaporin  
608 family, in order to alleviate the effects of water stress. Further research, involving e.g enhanced  
609 xylem delivery of exogenous ABA to embolized xylem vessels, will be needed to test this  
610 hypothesis.

611

612

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614

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618

619

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800 **Captions to figures**

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

805

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

810

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

818

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

822

823 **Fig. 5** Percent incidence of GO hits of up- and downregulated probes showing significant  
824 expression differences and reaching the  $\log_2 > 1$  threshold of expression ratio in the REC plants  
825 relative to IRR plants in all the time points of Exp. A (**a**), and in the REC plants relative to the IRR  
826 of Exp. B (**b**).

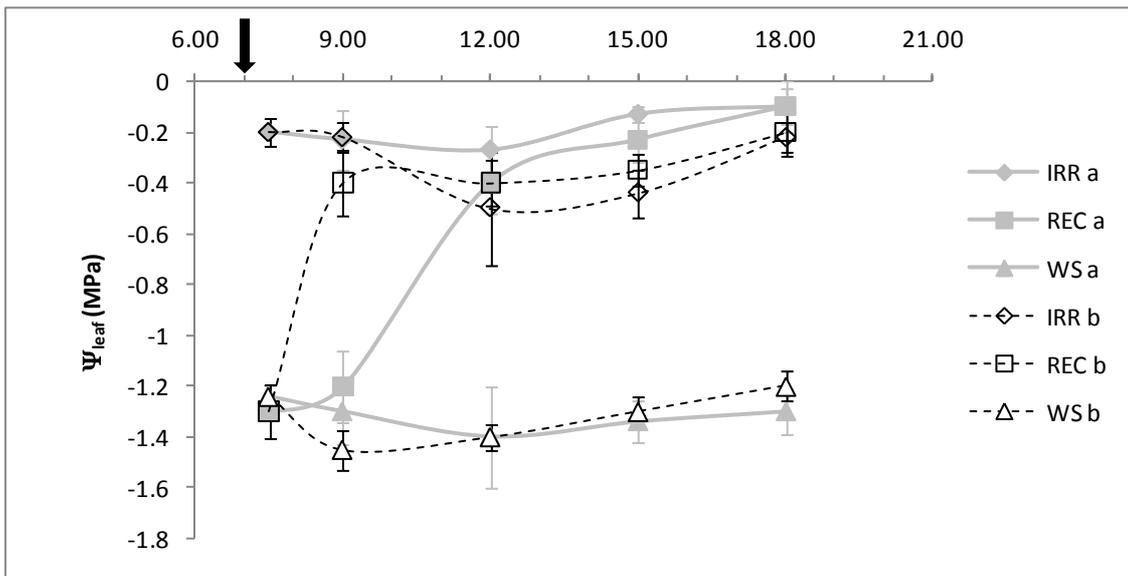
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828 **Fig. 6** Venn diagrams showing the number of probes whose expression was up- or downregulated  
829 (reaching the  $\log_2 > 1$  threshold of expression ratios) by water stress (WS) or rehydration (REC)

830 relative to IRR controls at all the time points in Exp. A (above left), and in Exp. B (above right),  
831 and by rehydration in the two experiments (REC A and REC B) (below).

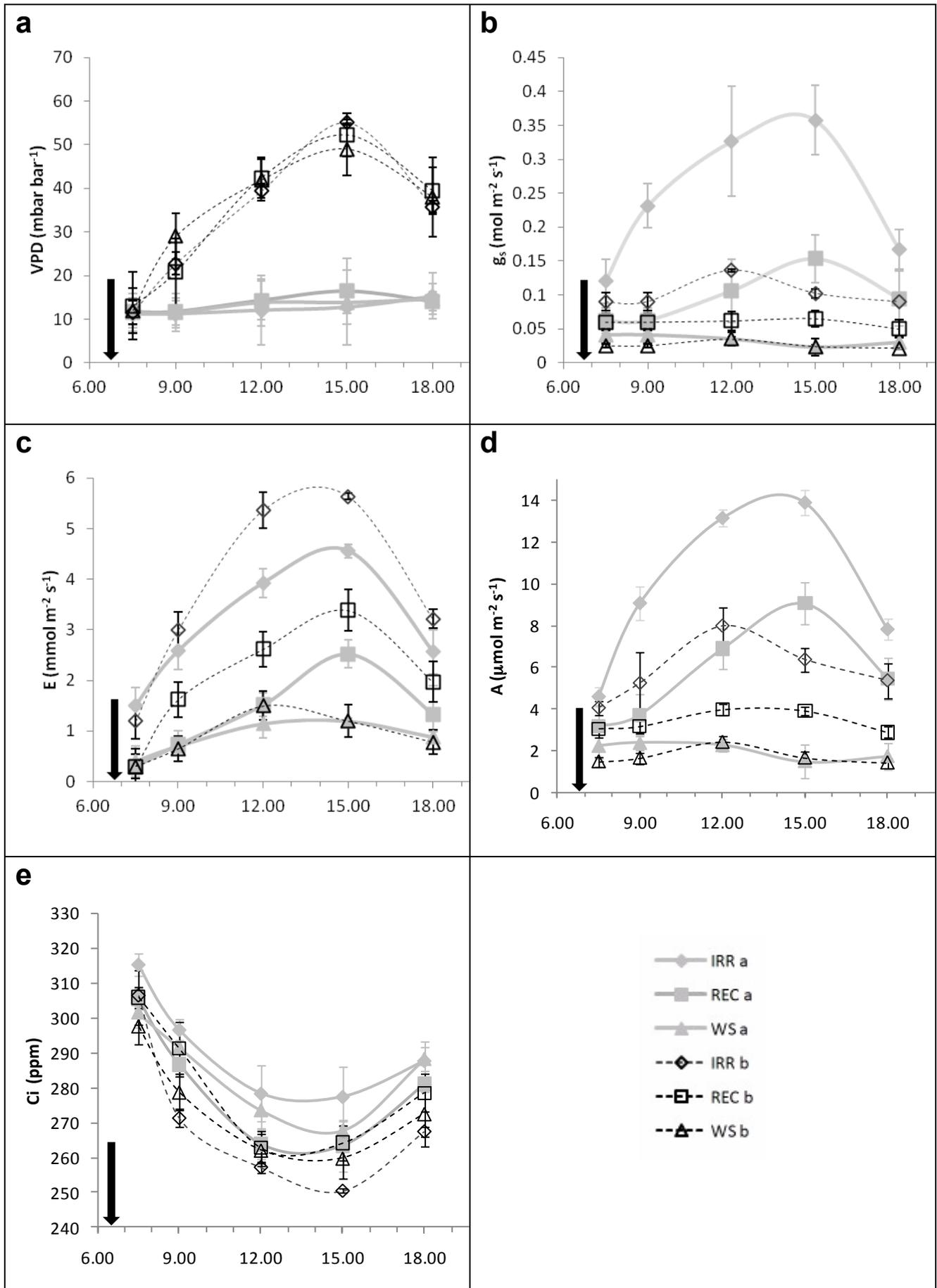
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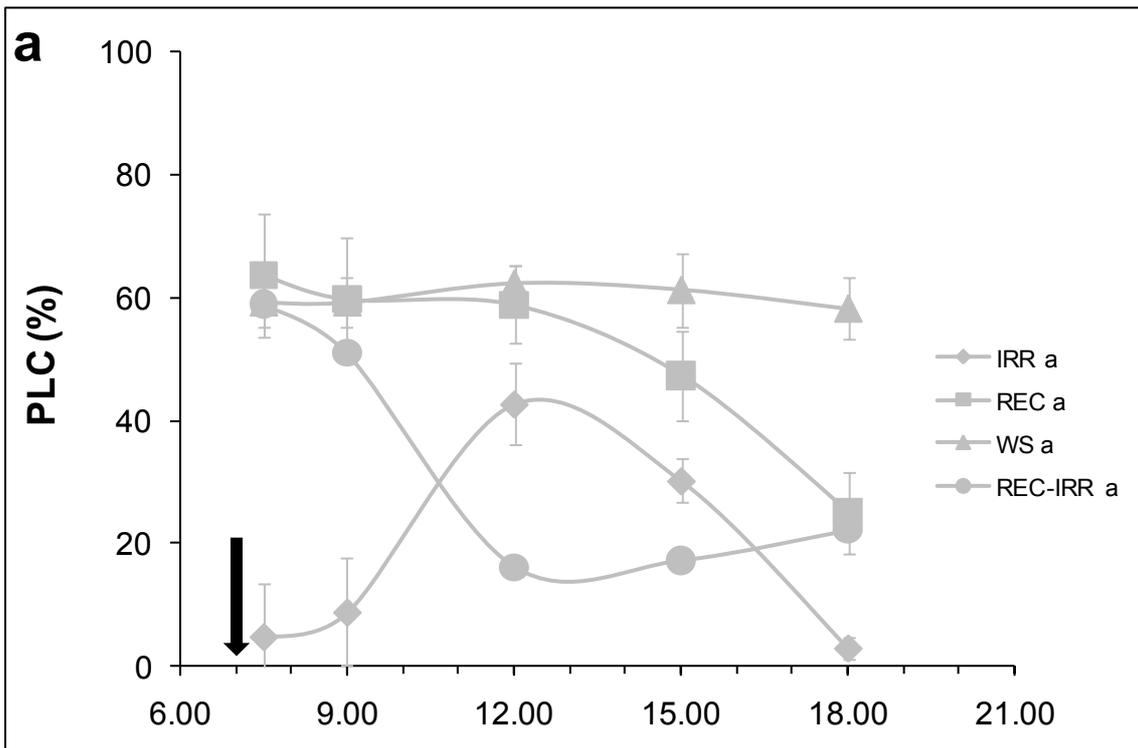
833 **Fig. 1**



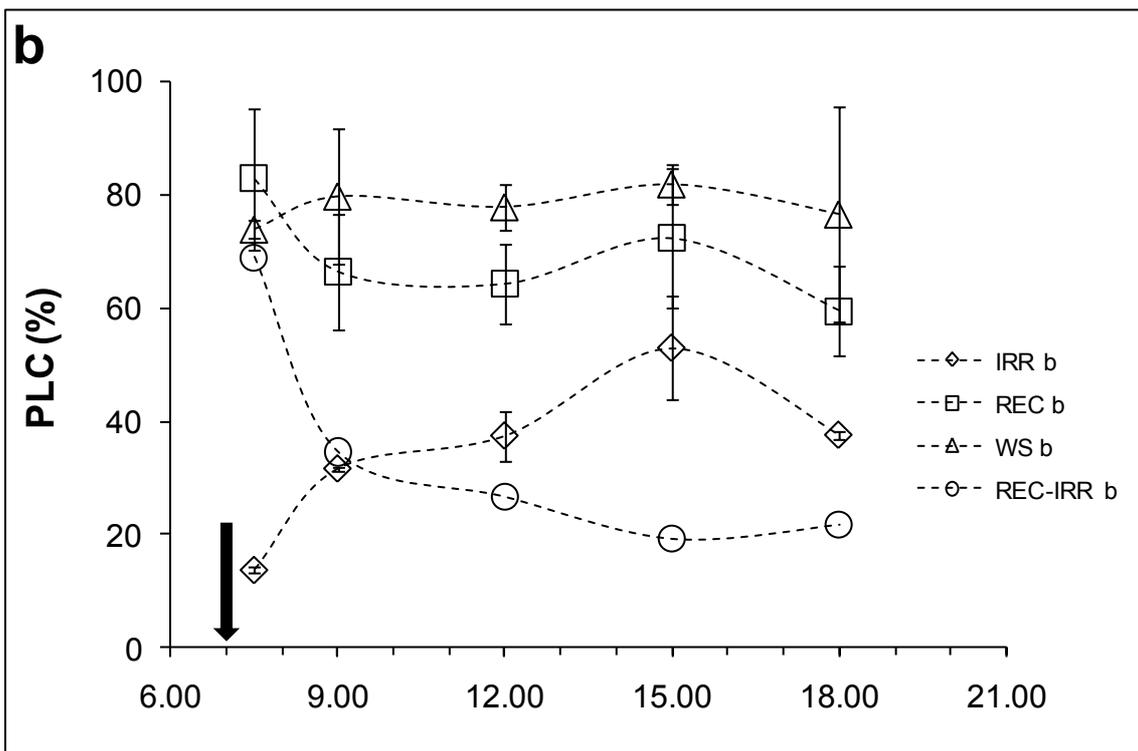
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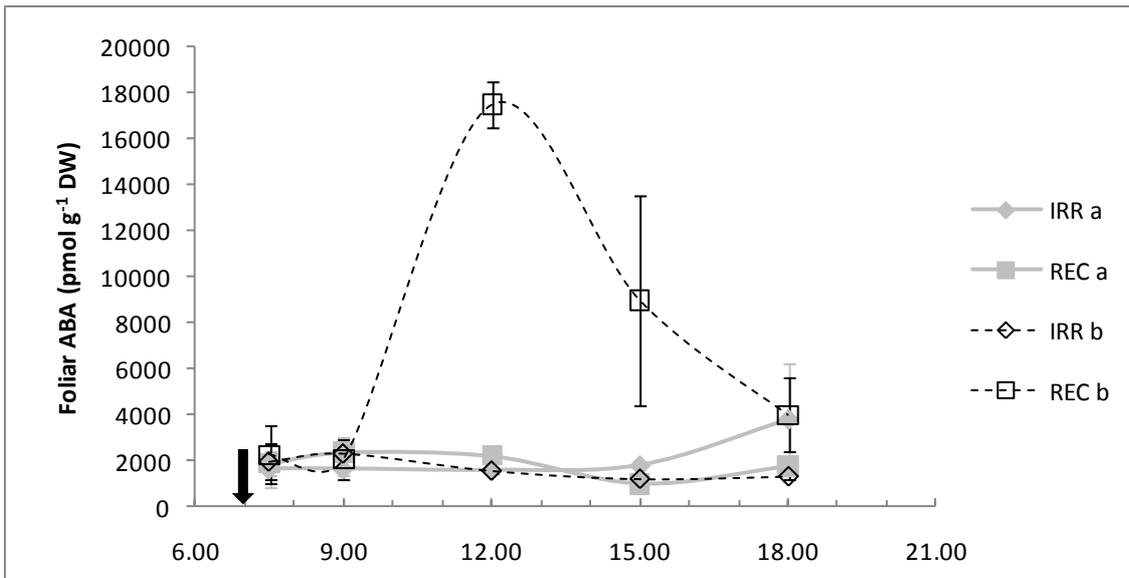
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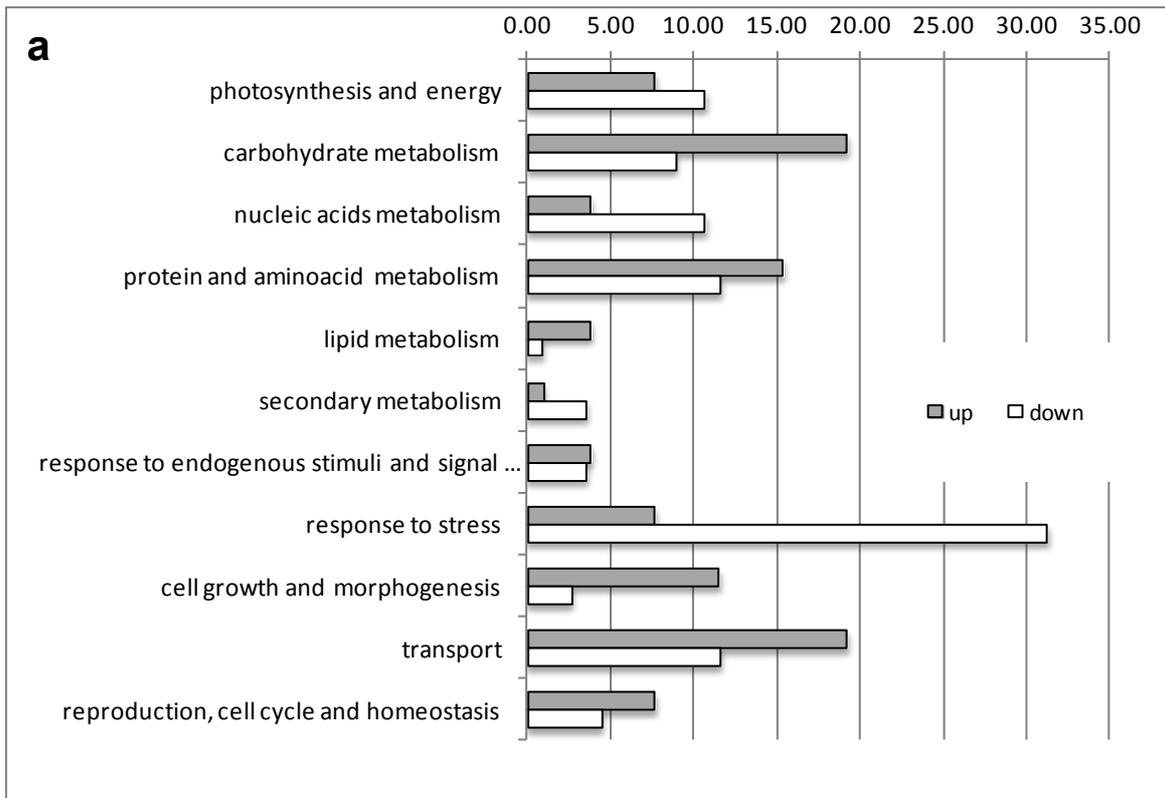
842 **Fig. 4**



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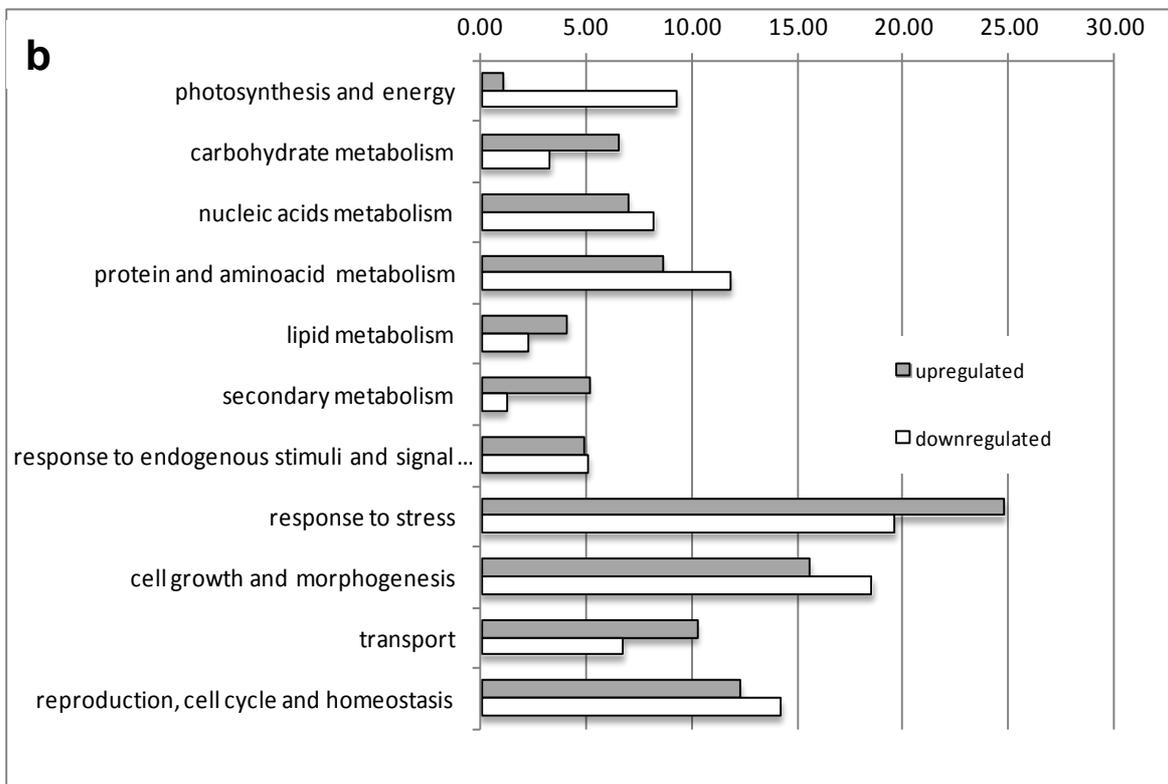
844 **Fig. 5**

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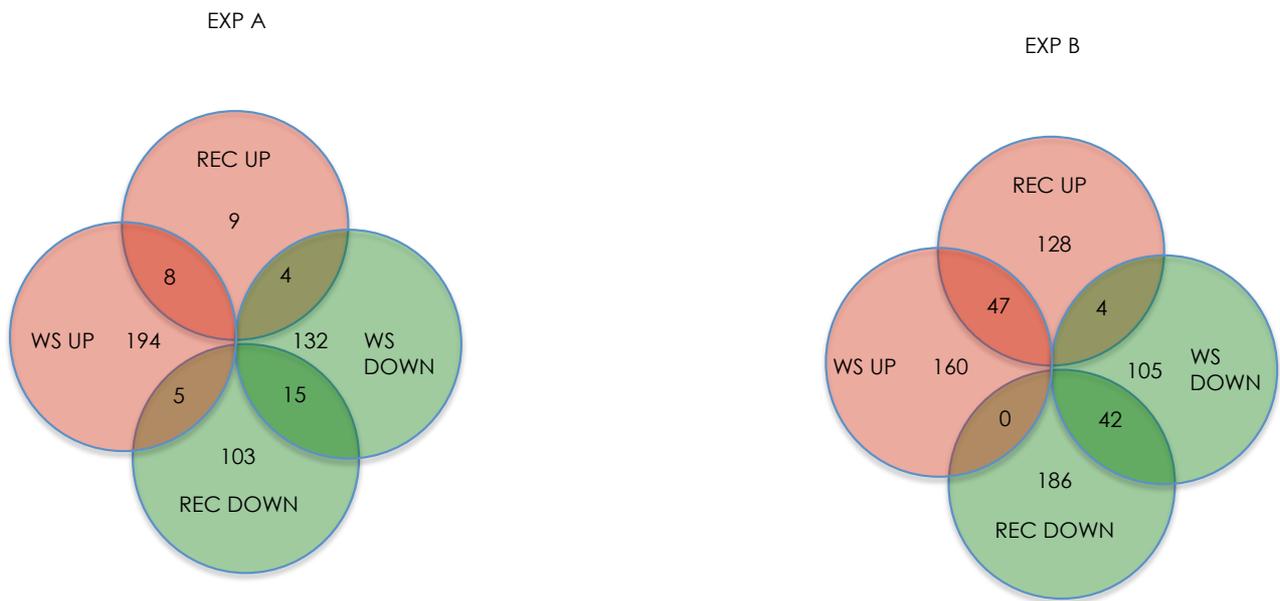
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850 **Fig. 6**  
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