1 **Insights into grapevine defense response against drought as revealed by biochemical,**
2 **physiological and RNA-Seq analysis**

3 Muhammad Salman Haider, Cheng Zhang, Mahantesh M. Kurjogi Tariq Pervaiz, Ting Zheng,
4 Chao bo Zhang, Chen Lide, Lingfie Shangguan, Jinggui Fang*

5 College of Horticulture, Nanjing Agricultural University, Tongwei Road 6, Nanjing 210095, PR.
6 China

7 *Corresponding author

8   Jinggui Fang

9   Email: fanggg@njau.edu.cn

10 **Abstract**

11 Grapevine is economically important and widely cultivated fruit crop, which is seriously
12 hampered by drought worldwide. It is necessary to understand the impact of glitches incurred by
13 the drought on grapevine genetic resources. Therefore, in the present study RNA-sequencing
14 analysis was performed using cDNA libraries constructed from both drought-stress and control
15 plants. Results yielded, a total of 12,451 differentially expressed genes (DEGs) out of which
16 8,022 genes were up-regulated and 4,430 were down-regulated. Further physiological and
17 biochemical analyses were carried out to validate the various biological processes involved in the
18 development of grapevine in response to drought stress. Results also showed that decrease in rate
19 of stomatal conductance in-turn decrease the photosynthetic activity and CO_2 assimilation rate in
20 the grapevine leaves and most ROS detoxification systems, including stress enzymes, stress-
21 related proteins and secondary metabolites were strongly induced. Moreover, various hormones
22 were known to be induced in the present study in response to drought. Overall the present study
23 concludes that these DEGs play both positive and negative role in drought tolerance by
24 regulating different biological pathways of grapevine. However our findings have provided
25 valuable gene information for future studies of abiotic stress in grapevine and other fruit crops.

26 **Keywords:** Transcriptome, Drought-stress, Grapevine, Chlorophyll, Secondary metabolites,

27 **Introduction**

28 Grapevine (*Vitis vinifera* L.) is an economically important crop, having 7.8 million hectares of
29 cultivated land with an annual production of 67.6 million tons worldwide [1]. The climate change
30 pattern has influential effects on the survival and productivity of grapevine. Thus, growth of
31 grapevine is consequently affected by abiotic stress, such as drought, salinity, etc. Drought has
32 deleterious effects on grapevine cultivation worldwide [2,3]. Globally, 45% of the agricultural
33 lands are under constant/ periodic water shortages [4], finally resulting in nearly 50% of yield
34 losses. Plants as being sessile organism are capable of making adaptive changes in physiology
35 and morphology that allow them to tolerate environmental stress but these adaptations are
36 inadequate to restore physiological water potential in the cell [5]. Plant response to these limited
37 water conditions is mediated by expression of numerous genes encoding stress-related proteins,

38 enzymes and metabolites functioning in the various pathways of cell metabolism [6]. The genes
39 induced under osmotic stress in plants are categorized into two groups such as, functional
40 proteins and regulatory proteins [7,8].

41   Water scarcity is not only threat for viticulture productivity, but also for wine quality [9,10].
42 Schultz proposed that an increase in environmental temperature due to rise in atmospheric CO_2,
43 is primary cause of water shortages for viticulture [11]. Grapevine possess the unique molecular
44 machinery which adjusts the flow of water to leaf and then to the atmosphere by vessel anatomy
45 [12], stomatal conductance [13] and aquaporin [14]. Consequently, the slow leaf and shoot growth,
46 elongation of tendrils, inhibition of internodes extension, leaf enlargement, decline of an average
47 diameter of xylem vessels and a minor stimulation in root growth under drought is observed in
48 grapevine [12].

49   RNA-seq is a novel technique that implicates deep-sequencing technology to achieve
50 transcriptomic profiling of both model and non-model plants. This approach enables researchers
51 to perceive novel genes in a single assay, allowing the detection of transcript information, allele-
52 specific gene expression and single nucleotide variants without the availability of ESTs and gene
53 annotations. Moreover, transcriptome data have also been used in characterizing large-scale
54 genes governing the complex interaction and metabolic processes of plant under stress [15]. In
55 addition, one step PCR enables researchers to predict the corresponding phenotype by detecting
56 the expression of genes before recording the morphological changes in plant. Thus, advantage of
57 this technique should be exploited in crop production, especially in countries with adverse
58 environmental conditions. we have successfully implicated this technique in our previous study
59 of different fertilization trials in grapevine [16]. However, the drought-regulated stress-response in
60 grapevine has not been studied in detail so far. Therefore, the aim of this study is to elucidate the
61 physiological responses of grapevine to drought stress, and further identify the DEGs in various
62 biological pathways. These results also provide the defense-related gene information, which can
63 be used for the development of drought-resistant grapevine cultivars.

Results

65   The sequence data obtained from the Illumina deep-sequencing was submitted to Short
66 Read Archive (SRA) database at NCBI under accession number SAMN04914490. After
67 filtering, raw data yielded 42.47 and 53.05 million clean reads in control and drought-stressed
68 leaf samples, respectively. The sequence alignment (soap2/SOAPaligner;
69 http://soap.genomics.org.cn) to the grapevine reference genome, allowed two base mismatches.
70 The total mapped reads (73.44%) were corresponding to unique (72.01%) and multiple (1.44%)
71 genomic positions (Supplementary: Table S1).

72   In current study, the sum of 12,451 DEGs was expressed under drought stress ($|\log_2 \text{Ratio}| \geq$
73 1) and false discovery rate (FDR $\leq$ 0.001); whereas, 8,022 (64.43%) were up-regulated and 4430
74 (35.57%) were down-regulated (Supplementary: Table S2).

Gene ontology (GO) and KEGG analysis of differentially-expressed genes

76   GO term mainly includes cellular components, molecular function and biological process.
77 A sum of 12,451 (72.11%) transcripts were annotated and classified into 51 functional groups,
78 including 21 in biological process, 16 in cellular component and 14 in molecular functions

79   (Supplementary: Table S3; Figure. S1). Under the biological process, out of 5994 transcripts,
80   the predominant transcripts found to be in metabolic process which includes 4537 transcripts
81   (75.7%; GO: 0008152), followed by cellular process consist of 3632 (60.6%; GO: 0009987) and
82   single-organism process involves 3185 transcripts (53.1%; GO: 0044699). Whereas, in cellular
83   component, out of 3940 reads the highest prevalence of transcripts were recorded in cell and cell
84   part with 3247 (82.4%; GO: 0005623 and GO: 0044464, respectively) transcripts, followed by
85   organelle" with 2358 (59.8% GO: 0043226) transcripts. Further 4291 and 3497 transcripts were
86   observed with the catalytic (71.3% GO: 0003824) and binding (58.1% GO: 0005488) activity
87   respectively in molecular function.

88   Several DEGs from the current study were subjected to KEGG annotation for further
89   characterization of transcripts, where 12,451 transcripts were allotted to 306 KEGG pathways.
90   The study revealed that the highest numbers of transcripts (2126) were involved in the metabolic
91   pathway (1257 up-regulated, 167 down-regulated), followed by biosynthesis of secondary
92   metabolites (out of 1160 transcripts; 681 were up-regulated, 479 were down-regulated), then 756
93   transcripts were recorded in plant-pathogen interaction pathway (241 up-regulated, 241 down-
94   regulated), while lowest transcripts (21) were recorded in sesquiterpenoid and triterpenoid
95   biosynthesis pathway in which 14 transcripts were up-regulated and 7 transcripts were down-
96   regulated (Supplementary: Table S4).

Chlorophyll degradation and photosynthetic competences under drought stress

98   The results of chlorophyll estimation unveil that 34.88% decrease of chl-a content in drought
99   treated grapevine leaf (0.28 ± 0.06 mg g^{-1}) when compared with that of control plant leaf ($0.43 \pm$
100   0.11 mg g^{-1}). Similarly 21.92% decrease in chlb of leaf exposed to drought stress (0.57 ± 0.04 mg
101   g^{-1}) compared to control leaf (0.73 ± 0.06 mg g^{-1}). In the same way, photosynthesis rate was also
102   decreased by 32.20% in drought treatment (16.08 ± 0.75 µmole m^{2-} sec^{-1}) when compared to
103   control (23.67 ± 0.81 µmole m^{-2} sec^{-1}). Moreover, stomatal conductance and CO_2 assimilation
104   rate also showed significant reduction by 40.00% (0.11 ± 0.04) and 44.44% (5 ± 0.03) in drought
105   treated grapevine leaves compared to that of control (Table 1).

106   In the grapevine transcriptome, 29 DEGs involving in chlorophyll metabolic pathway responded
107   differently to drought stress compared with control, of which 18 transcripts were up-regulated
108   and 11 transcripts were down-regulated. However, out of 25 transcripts functioning in chl
109   synthesis and degradation, 9 transcripts involved in chla synthesis (Glutamate tRNA Ligase;
110   Radical S-adenosyl methionine domain-containing protein1;   Protoporphyrinogen oxidase;
111   Dehydrogenase/reductase SDR family member; Protochlorophyllide oxidoreductase, and four
112   transcripts of Short chain dehydrogenase, TIC32) were significantly up-regulated; whereas, 7
113   transcripts (2 transcripts of HemA, Glutamate tRNA reductase 1; Proporphynogen oxidase 1; 2
114   transcripts of CHLH, Magnesium chelatase H subunit; Protochlorophyllide oxidoreductase and
115   Short chain dehydrogenase) were significantly down-regulated. Meanwhile, in the chl cycling
116   process Chlorophyllide a oxygenase, (CAO) was significantly down-regulated, but Chlorophyll
117   (ide) b reductase NYC1 (CBR) was up-regulated by the drought treatment. Whereas,
118   Chlorophyllase-II, Pheophorbide a oxygenase, and Protochlorophyllide-dependent translocon
119   component 52, were significantly up-regulated and 3 transcripts of Chlorophyllase-I, were
120   down-regulated during the chl degradation process (Table 2, Figure, 1,). The expression level of
121   VIT_08s0007g08540.t01 (307.93-106.65 RPKM) and VIT_19s0014g03160.t01 (1360.37-307.58
122   RPKM) revealed high profusion in chla synthesis pathway. Moreover, in the phytochromobilin

123 synthesis, the expression of Ferrochelatase-2 (VIT_07s0031g03200.t01, $|log_2FC|$ = 3.032), Heme
124 oxygenase 1 (VIT_11s0016g05300.t01, $|log_2FC|$ = 2.403), Heme oxygenase 2
125 (VIT_18s0001g11040.t01, $|log_2FC|$ = 2.249) and Phytochromobilin:ferredoxin oxidoreductase
126 (VIT_06s0009g03770.t01, $|log_2FC|$ = 1.705) was also induced by the drought stress
127 (Supplementary: Table S5).

128 In grapevine transcriptome, a sum of 23 DEGs related to photosynthesis pathway, including PSII
129 (5), PSI (2), cytochrome b6-f complex (4), photosynthetic electron transport (4), F-type ATPase
130 (4), photosynthesis-antenna proteins (4) were recorded sensitive to drought stress. In PSII (5
131 DEGs), which includes psbB (2), psbCs (2) and psbW (1) and all 5 DEGs were found to be
132 significantly down-regulated. psbC (VIT_00s0396g00010.t01, 280.56 - 1.32 RPKM) possessed
133 the high expression abundance . Moreover, two psaBs in PSI and two transcripts related to
134 cytochrome b6-f complex (petA and petC) revealed significant reduction in their expression
135 levels, perhaps two transcripts of petC were found to be increased with control group . Similarly,
136 4 genes involved in the photosynthetic electron transport unveiled that, two transcripts of petF
137 (VIT_12s0035g00270.t01 and VIT_06s0080g00410.t01) were down-regulated and two
138 transcripts of petH (VIT_04s0023g03510.t01 and VIT_10s0003g04880.t01) were up-regulated
139 when compared with control. In addition, the F-type ATPase-related genes (ATPF1B, ATPF1A,
140 ATPF1G and ATPF0C) and photosynthesis-antenna proteins-related genes (LHCB1, LHCB2,
141 LHCB3 and LHCB6) were found to be significantly down-regulated in drought treated leaves
142 (Table 2, Supplementary: Table S6).

143 **ROS system under drought stress**

144 The Malondialdehyde activity was increased significantly (60.93%) in drought treatment (8.61 ±
145 0.25 nmol g^{-1}) compared to control (5.35 ± 0.21 nmol g^{-1}). A significant increase was observed in
146 the activity of superoxide dismutase (75.16%), peroxidase (140.81%) and catalase (200.79%) in
147 drought responsive grapevine leaves in comparison with control (Table 1). In transcriptomic
148 analysis, one NADPH respiratory oxidase and five amine oxidases functioning in the ROS
149 synthesis process were significantly up-regulated in drought treated grapevine leaf samples. In
150 ROS scavenging system, 60 DEGs were identified that were categorized into Fe superoxide
151 dismutase (2 transcripts), peroxidase (6 transcripts), catalase (3 transcripts), glutathione-
152 ascarbate cycle (9 transcripts), glutathione peroxidase (1 transcript), glutathione S-transferaze
153 (26 transcripts), peroxiredoxin/thioredxin pathway (8 transcripts), alternative oxidases (3
154 transcripts) and polyphenol oxidase (2 transcripts) (Figure 2; Supplementary: Table S7).

155 In our findings, two Fe-SODS were up-regulated, but both genes showed low expression
156 abundance. In contrast, 2 Cu/Zn-SOD were significantly down-regulated ($|log2FC|$ < 1), CAT (3
157 transcripts) and POD (2 transcripts) were significantly up-regulated. All three up-regulated CAT
158 transcripts (VIT_18s0122g01320.t01, from 2888.01 to 358.79 RPKM; VIT_00s0698g00010.t01,
159 from 428.21 to 106.90 RPKM; VIT_04s0044g00020.t01, from 767.21 to 263.73 RPKM) showed
160 high expression abundance; whereas, all up/down-regulated POD genes showed moderate to low
161 expression abundance. Furthermore, 9 GSH-AsA (5 up-regulated, 4 down-regulated), 27 GPX-
162 pathway (23 up-regulated, 4 down-regulated), eight Prx/Trx (5 up-regulated, 3 down-regulated),
163 three AOX (2 up-regulated, 1 down-regulated) and two PPO (down-regulated) genes were
164 identified in response to drought stress, (Table 3. Supplementary: Table S7).

165 **Plant hormone signal transduction pathway under drought stress**

166   The hormonal level, including auxin was increased in drought treatment (1.626 ± 0.03 ng g^{-1}
167   FW) compared to control (1.373 ± 0.02 ng g^{-1} FW). Similar trend was observed in abscisic acid
168   that is 0.908 ± 0.01, and 0.257 ± 0.01 ng g^{-1} FW for drought and control treatments, respectively.
169   In the same way jasmonic acid in drought treatment sample was 1.67 ± 0.05 ng g^{-1} FW, whereas,
170   in control it was found to be 1.451 ± 0.03 ng g^{-1} FW. Further gibberellic acid (GA) in treated and
171   control sample was recorded to be 1.671 ± 0.02, and 1.53 ± 0.02 ng g^{-1} FW, respectively. Alike
172   brassinosteroid also showed 1.091 ± 0.01, and 1.073 ± 0.01 ng g^{-1} FW for drought and control
173   treatment samples, respectively (figure 3). In grapevine transcriptome, several DEGs related to
174   AUX, GA, ABA, JA, ET (ethylene), and BR were found in signal transduction pathways in
175   drought stressed grapevine leaves. Under AUX signaling, three genes (down-regulated) related
176   to auxin transport, eleven auxin response factors (7 up-regulated and 4 down-regulated) involved
177   in the transcriptional repressors were detected. Moreover, fifteen genes in auxin induced and
178   responsive proteins (2 up-regulated and 13 down-regulated), six IAA synthetase (GH3; 1 up-
179   regulated and 5 down-regulated) and seventeen genes related to auxin and IAA induced proteins
180   (SAUR; 5 up-regulated and 12 down-regulated) were perceived in grapevine under drought
181   stress . Two natural receptors were up-regulated while four DELLA proteins were down-
182   regulated in the GA under drought stress. Moreover, three ABA responsive proteins (down-
183   regulated), two SNF1-related protein kinases 2 (SnRK2; up and down-regulated), three PP2C
184   group (up-regulated) genes and six transcription factors (ABF, up-regulated) were involved in
185   abscisic acid pathway. Six transcripts of jasmonate-ZIM-domain proteins (one up-regulated and
186   5 down-regulated) and single jasmonoyl isoleucine conjugate synthase 1 (up-regulated) were
187   found in JA hormonal signaling. Moreover, 12-oxophytodienoate reductase 2-like (up-regulated),
188   linoleate 13S-lipoxygenase 2-1 (up-regulated) and allene oxide synthase (down-regulated) were
189   identified in JA pathway under drought stress. Three ethylene-responsive transcriptional factors
190   (3 up-regulated) being crucial to ET, five ethylene response factor (down-regulated) and three
191   ACC oxidases (up-regulated) were perceived grapevine leaf tissue responding drought stress. In
192   BRs, two transcripts related to BRASSINOSTEROID INSENSITIVE1 (up-regulated), ten
193   (down-regulated) brassinosteroid-regulated proteins (BRU1) and 9 (down-regulated) D-type
194   cyclins were functioning in plant hormone signal transduction pathway under drought stress
195   conditions (Supplementary: Table S8).

Proline metabolism under drought stress

197   The proline level showed significant increase in grapevine leaves responding to drought stress
198   (1.711 ± 0.05 ng g^{-1} FW) as compared with control plant leaves (1.624 ± 0.04 ng g^{-1} FW; Table
199   1). In transcriptomic analysis, a total of 18 DEGs, including pyroline-5-carboxylate synthetase ,
200   proline dehydrogenase, Proline methyltransferase ☐-Glutamyl kinase, Glutamic-☐-semialdehye
201   dehydrogenase, Pyrroline-5-carboxyate dehydrogenase, Prolyl hydroxylase (4 transcripts),
202   Acetyl-CoA: glutamate N-acetyl transferase 2 transcripts), N-Acetylglutamate kinase, Acetyl
203   glutamic-☐-semialdehyde dehydrogenase, Acetyl ornithine aminotransferase, Acetyl ornithine
204   deacetylase (2 transcripts), Arginino succinate lyase (ASL) and Arginase were significantly up-
205   regulated functioning in the proline synthesis and metabolism pathway in drought treatment
206   compared to control (Figure, 4, Supplementary: Table S9).

Biosynthesis of secondary metabolites under drought stress

208 In transcriptomic study, 73 secondary metabolites related genes linked with shikimate acid (9),
209 alkaloid (2), anthocyanin (33), lignin (21) and terpenoid (8) were recognized under drought
210 treated grapevine leaves.

211 Shikimate acid (SA) pathway possessed one up-regulated 3-deoxy-D-arabino-heptulosonate-7-
212 phosphate synthase 03 , two down-regulated 3-dehydroquinate dehydratase/shikimate
213 dehydrogenase , one down-regulated shikimate kinase one up-regulated chorismate synthase 1 ,
214 two down-regulated anthranilate phosphoribosyltransferase (AnPRT) and both up-regulated
215 indole-3-glycerol phosphate synthase (IGPS) and tryptophan synthase beta chain 1 (TS1),
216 respectively All SA genes have moderate transcript abundance.

217 In alkaloid biosynthetic pathway, genes related to strictosidine synthase 3 and D-amino-acid
218 transaminase were down-regulated . Out of 33 genes in anthocyanin biosynthesis, 8 genes
219 related phenylalanine ammonia-lyase (4-up-regualted and 4-down-regulated), one trans-
220 cinnamate 4-monooxygenase (down-regulated), two 4-coumarate--CoA ligase-like 9 (up and
221 down-regulated), 13 stilbene synthase (6 up-regulated and 7 down-regulated), 3 flavonol
222 synthase/flavanone 3-hydroxylase (one up-regulated and 2 down-regulated), one 1-
223 aminocyclopropane-1-carboxylate oxidase 5 (down-regulated), two dihydroflavonol-4-reductase
224 (down-regulated), one anthocyanidin reductase (up-regulated) and one anthocyanidin 3-O-
225 glucosyltransferase 2 (down-regulated) were observed, (Table 4, Figure 5, Supplementary: Table
226 S10).

227 In grapevine transcriptome, 21 differentially expressed genes were identified in lignin
228 biosynthesis, which were involved in the drought stress. It includes; 9 up-regulated genes related
229 to shikimate O-hydroxycinnamoyltransferase, aldehyde 5-hydroxylase, two caffeoyl-CoA O-
230 methyltransferase, cinnamoyl-CoA reductase 1, cinnamyl alcohol dehydrogenase 1, two
231 peroxidase and laccase; whereas, 12 DEGs were down-regulated including, two caffeic acid 3 O-
232 methyltransferase, one cinnamoyl-CoA reductase 1, five peroxidase, three laccase transcripts.

233 Further, eight genes were identified to be involved in terpenoid biosynthesis from which
234 hydroxymethylglutaryl-CoA synthase, 1-deoxy-D-xylulose 5-phosphate reductoisomerase,
235 isopentenyl diphosphate isomerase II and terpene synthase were up-regulated, while
236 hydroxymethylglutaryl-CoA synthase, 1-deoxy-D-xylulose-5-phosphate synthase and two
237 squalene epoxidase were down-regulated in drought-stressed grapevine leaves (Table 4, Figure
238 5, Supplementary: Table S10).

239 **Heat shock protein (HSP) and pathogenesis-related protein (PR) in response to drought**
240 **stress**

241 The results revealed that 48 DEGs were identified in HSPs, including one HSP101 (down-
242 regulated), three HSP90 (1 up-regulated and 2 down-regulated), two HSP70 (1 up-regulated, 1
243 down-regulated), eighteen sHSPs (12 up-regulated and 6 down-regulated), twenty other HSP
244 genes (17 up-regulated and 3 down-regulated) and heat-stress transcription factors (4 up-
245 regulated and 1 down-regulated) . The high molecular weight HSPs (HMW HSPs), including
246 HSP90s and HSP70s were also found to be up-regulated in our findings. One up-regulated
247 transcript of HSP70s was expressed at higher abundance level (VIT_17s0000g03310.t01; 650.81
248 to 532.18 RPKM), compared with other HMW HSPs. The up-regulated,
249 VIT_16s0098g01060.t01 (from 706.59 to 1.98 RPKM) from sHSPs and

250 VIT_14s0060g01490.t01 (from 363.93 to 355.88 RPKM) from other HSPs, expressed at
251 moderate abundances, but remaining sHSPs, other HSPs and heat-stress transcription factors
252 expressed at lower abundances (Table 5, Supplementary: Table S11).

253 In this study, 72 transcripts were identified as differentially-expressed genes, including ten
254 pathogenesis-related protein PR-1 (4 up-regulated, 6 down-regulated), nine Beta-1,3-glucanase
255 (PR2; 4 up-regulated, 5 down-regulated), nineteen chitinase (4 up-regulated, 15 down-regulated),
256 fourteen thaumatin-like protein (PR5; 6 up-regulated, 8 down-regulated), four Pathogenesis-
257 related protein 10 (2 up-regulated, 2 down-regulated), ten non-specific lipid-transfer protein
258 (PR14; 5 up-regulated, 5 down-regulated), four Germin-like protein 2 (2 up-regulated, 2 down-
259 regulated) and two pathogenesis-related transcription factors (2 down-regulated) to code disease
260 resistance proteins. Conversely to HSPs, most of the PR showed down-regulation in grapevine
261 leaves under drought stress. Moreover, 4 up-regulated transcripts, including PR1
262 (VIT_03s0088g00890.t01, $|log_2FC|$ = 8.75), chitinase (VIT_05s0094g00320.t01, $|log_2FC|$ =
263 8.29), thaumatin-like protein (VIT_02s0025g04290.t01, $|log_2FC|$ = 3.84) and Pathogenesis-
264 related protein 10 (VIT_05s0077g01600.t01, $|log_2FC|$ = 8.31) were only expressed in treatment
265 group. Additionally, ten dirigent proteins (3 up, 7 down-regulated) and thirteen proline related
266 proteins (6 up, 6 down-regulated) were also recorded from this study (Table 5, Supplementary:
267 Table S11).

qRT-PCR validation of DEGs from Illumina RNA-Seq

269 In order to investigate the accuracy and reproducibility, 16 DEGs were selected from RNA-Seq
270 results for quantitative real-time PCR, these transcripts represent all the major up/down-regulated
271 functions that were identifies in our transcriptome data including, metabolism, hormone
272 signaling, disease resistance and regulatory proteins. The gene function, primer sequence,
273 RPKM, Log_2 values and qRT-PCR results are presented in Figure. 6; Supplementary: Table S12.
274 The qRT-PCR findings of 16 (8 up-regulated and 8 down-regulated) selected genes were
275 consistent with the RNA-seq results, revealing the accuracy and reliability of our RNA-seq
276 results.

Discussion

278 Drought stress suppresses the plant growth by inhibiting many physiological processes of plants.
279 Cholorphylls (Chls) are the principal light-absorbing pigments and key components of
280 photosynthesis in plants. The physiological and transcriptomic studies of grapevine leaves
281 responding to drought stress have revealed that chl contents were remarkably decreased which
282 in-turn inhibited the photosynthetic activity. Similarly decrease in chl content was reported in
283 corn and chickpea in response to drought stress [17,18]. Moreover, transcriptomic data
284 demonstrated that drought stress inhibited the chl biosynthesis process by suppressing the
285 activity of key enzymes such as, HemA (Glutamyl-tRNA reductase 1) and CHLH (Magnesium
286 chelatase H subunit), which play key role in chla synthesis process [19]. Furthermore in chl cycle,
287 the oxygenation reactions of chlorophyll(ide) a to chlorophyll(ide) b are catalyzed by
288 chlorophyllide a oxygenase (CAO) [20], whose activity was also decreased under drought stress,
289 suggesting the obstructed process of chl cycle. In contrast, the chlorophyll(ide) b to a conversion
290 is catalyzed by chlorophyll(ide) b reductase NYC1 (CBR) and its activity was up-regulated,
291 suggesting that chl cycle process was also suppressed by the drought treatment [21]. Furthermore,
292 PAO (pheophorbide a oxygenase) is regarded as an important chl catabolic enzyme [22,23] and

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293 participated well in chl degradation process as its activity was increased under drought stress
294 (Figure.1 Supplementary: Table S5). [24]Buchert and [25]Du have investigated the role of PAO as an
295 important chl degradation enzyme during senescence of broccoli and banana, respectively.

296 Meantime, the photosynthetic activity, stomatal conductance and CO_2 assimilation rate was
297 significantly decreased in grapevine leaves under drought stress as compared to control. Similar
298 findings have also been reported in grapevine under Cu and drought stresses[26,27]. Moreover, the
299 photosynthesis-related genes, involved in PSII, PSI, cytochrome b6-f complex, photosynthetic
300 electron transport, F-type ATPase and photosynthesis-antenna proteins were significantly down-
301 regulated in drought-induced grapevine leaves, but the extent of light-harvesting proteins (CP47,
302 CP43), which binds the chla molecules was down-regulated by the drought stress
303 (Supplementary: Table S6). Perhaps, PsaB is regarded as the heart of PSI that binds P700 special
304 chlorophyll pair [28] was also down-regulated under drought stress in our findings. Finally, drought
305 stress gradually decreased the activities of PSII electron transport and light-harvesting complex
306 (photosynthesis-antenna proteins). Available literature anticipated that stomatal closure reduced
307 the CO_2 absorption which limits the photosynthetic activity in plants under drought stress
308 environment[29-31]. Our findings on photosynthesis phenomenon at physiological and
309 transcriptomic level suggested that drought stress definitely affected the primary photosynthesis
310 metabolic process, and the decline in photosynthesis process was connected with the chlorophyll
311 degradation.

312 ROS is the universal response of the plants against any type of environmental stress to prevent
313 oxidative damage. Several studies have already been conducted on malondialdehyde under
314 oxidative stress in different crops such as, wheat (*Triticum aestivum*) and oilseed rape (*Brassica
315 napus*), proposed that MDA contents were induced by drought stress [32-34]. On the contrary, plants
316 have the ability to accumulate the level of antioxidative enzymes to confer the severity of
317 drought stress and similar investigations in olive [35] and wheat [33] support our findings of
318 increased activity of ROS enzymes and MDA. The results of transcriptomic investigation
319 showed that, one NADPH oxidase and five amine oxidases were significantly up-regulated,
320 while both play key role in the ROS synthesis and accumulation under various kind of stress
321 environments [36]. SODs are regarded as first line of defense against ROS which have two
322 isozymes Fe-SOD and Cu/Zn-SOD in plant chloroplast [36]. It is worth mentioning that Fe-SODs
323 was up-regulated, but Cu/Zn-SODs was down-regulated, which are in agreement with our
324 previous findings in grapevine under Cu stress conditions [26]. Other enzymes, including CAT,
325 POD, GSH-AsA cycle, PPO, GST, AO, MDHAR, DHAR and GR also possess the drought-
326 responsive antioxidative defense system in grapevine [37]. Perhaps, non-enzymatic antioxidants
327 such as, glutathione and proline also enhanced the ROS level in grapevine in response to
328 drought-stress, which is consistent with the ROS scavenging system investigated in the *V.
329 vinifera* and *S. lycopersicum* under drought stress [38,39]. Generally, ROS related analytical and
330 transcriptomic findings present the broad spectra to understand their role at cellular level in
331 response to drought stress.

332 Drought stress causes dehydration in plant cells. Plant hormones, such as abscisic acid, auxin,
333 Gibberellin, ethylene, jasmonic acid and brassinostroid accumulate under dehydration condition
334 and play important role of stress tolerance in plants [40]. In *Arabidopsis*, ABA activates the
335 subclass III protein kinases of SnRK2 family, which further facilitate the regulation of stomatal
336 conductance to regulate plant water status through guard cells [41,42], favor our findings of

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337 increased activity of SRK2I protein kinase under drought stress in grapevine leaves. The
338 regulation of PP2C genes during the drought stress in grapevine leaves proposed that PP2C has
339 its primary role in stress tolerance, especially in regulating ABA response [43]. The AUX gene
340 family includes early response AUX genes, Aux/IAA, GH3 and SAUR and the regulators of
341 AUX genes, ARF, while their activities were down-regulated in our findings. Wang et al. [44]
342 investigated the AUX gene family in sorghum (*Sorghum bicolor*) and specified that most of
343 these genes were induced by the exogenous application of IAA under drought stress conditions.
344 Moreover, GA activity and the accumulation of DELLA proteins was up-regulated by the
345 drought stress, while similar findings in *Arabidopsis* have suggested that DELLA proteins
346 restrain the plant growth to promote survival of plant under drought stress [44]. JA biosynthesis and
347 signaling together with ABA and other hormones have been extensively studied in many crops.
348 In current investigations, JA amino acid conjugate (JAR1) was significantly up-regulated, while
349 JAR1 are enduringly present in the plant leaves and together with ABA induce the stomatal
350 closure under osmotic stress, have been extensively studied in *Arabidopsis* [45]. Interestingly,
351 jasmonate-zim domain proteins (JAZ) were significantly down-regulated, which was observed
352 up-regulated in another study in rice [46], suggesting the severity of drought stress in grapevine
353 leaves. Moreover, the activity of AOS and LOX were significantly increased, which is similar
354 with the findings of Leng et al. [36] in *V. vinifera*. Ethylene is regarded as stress hormone because
355 its synthesis is induced under different oxidative environments. Under drought stress, the
356 synthesis of ethylene precursor 1-aminocyclopropane-1-carboxylate oxidase was up-regulated in
357 grapevine, which stimulates plant development and functioning by inducing the diffusion
358 possibility of ABA to its active site [47,48]. Furthermore, the expressions of the ethylene-related
359 regulatory genes (ETR1 and CTR1) were intensely increased in our findings, suggesting their
360 key role in ethylene biosynthesis as described by Schachtman and Goodger [49]. BRs are the only
361 plant steroids, which induce the expression of many genes, especially during stress
362 environments. Brassinosteroid Insensitive 1 (BRI1) was significantly up-regulated in our
363 findings, which is known to play key role in plant growth, morphogenesis and response to
364 drought stress. Feng, et al. [50] created RNAi mutants for bdBRI1 in *Brachypodium distachyon* and
365 suggested that this gene produces a dwarf phenotype with enhanced tolerance against drought
366 stress. BR signal transduction, from cell surface perception to activation of specific nuclear
367 genes will be interesting to investigate in the future.

368 Plants cope with environmental stress by the accumulation of certain compatible osmolytes such
369 as, proline, which is known to confer the drought tolerance in plants [51] and up-regulation of all
370 the genes related to proline metabolism is the clear evidence of grapevine tolerance in our study.
371 Proline biosynthesis commenced with the phosphorylation of glutamate, which then converted
372 into gulatamic-☐-semialdehyde by Pyroline-5-carboxylate synthetase (up-regulated). Similarly,
373 arginine is converted into orthinine by arginase (up-regulated) and then into GSA by the
374 ornithine-δ-aminotransferase (not-detected). GSA is then converted into pyrroline 5-carboxylate
375 (P5C) by impulsive cyclization. Finally, proline is synthesized from the P5C by P5C reductase
376 (P5CR) enzyme [51,52]. In proline degradation pathway, proline is re-converted into P5C by Proline
377 dehydrogenase (PDH; up-regulated) and then into glutamate by Pyrroline-5-carboxylate
378 dehydrogenase (P5CDH; up-regulated). Thus PDH and P5CDH are believed to be most
379 important enzymes in proline degradation to glutamate [53,54]. Hence, proline metabolism may
380 regulate the gene expression during the drought stress.

381 In higher plants, accumulation of various secondary metabolites such as, amino acids,
382 carbohydrates and lipids occur when plant is subjected to environmental stress[55]. Shikimate
383 pathway not only act as bridge between central and secondary metabolism, but also serve as
384 precursor for other secondary metabolites [56]. Additionally, Tyr is a precursor of IAA and initiate
385 the synthesis of indole alkaloids and isoquinoline alkaloids, which prevent plants from oxidative
386 stress [57]. Phe is considered as precursor of secondary metabolites family and PAL participates in
387 phenylpropanoid biosynthesis; a key step towards biosynthesis of stilbenes, flavonoids, lignins
388 and various other compounds [58]. STS (stilbene synthase) catalyzes the initial step of flavonoid
389 biosynthesis pathway, which has the protective function during the drought stress [59]. Overall, 4
390 PAL and 6 STS were significantly up-regulated in our findings, proposing the innate link with
391 drought stress. The respective, up and down-regulation of 1-deoxy-D-xylulose 5-phosphate
392 reductoisomerase and 1-deoxy-D-xylulose-5-phosphate synthase can act as rate limiting enzymes
393 in MEP pathway, also found in cu-stressed grapevine leaves [26]. Dimethylallyl diphosphate and
394 isopentenyl diphosphate are the universal 5 carbon precursors found in terpenoid synthesis. It has
395 been reported that one isopentenyl-diphosphate isomerase II can catalyze isopentenyl
396 diphosphate to form dimethylallyl diphosphate and one terpene synthase [60,61], while both were
397 up-regulated in our findings. The down-regulation of most of the genes related to anthocyanin,
398 lignin and terpenoid biosynthesis have elucidated the negative role of drought stress on
399 accumulation of secondary metabolites in grapevine leaves.

400 HSPs are ubiquitous stress-related proteins that act as molecular chaperone, HSP members
401 participate in the protein synthesis, folding, aggregation and transportation from cytoplasm to
402 different intracellular compartments [62,63]. In current study some high molecular weight HSPs
403 (HSP101, HSP90 and HSP70) were down-regulated, but most of the genes related to small
404 HSPs (sHSPs; 16-30kDa), other HSPs and heat stress transcription factors (HSFs) were up-
405 regulated. In addition, pathogenesis-related (PR) proteins are derived from plant allergens
406 and act as defense-responsive proteins by increasing their expression under pathogen attack
407 and variable stress environments. Depending on the functions and properties, PR-proteins
408 are classified into 17 families such as, beta-1,3-glucanases, chitinases, thaumatin-like
409 proteins, peroxidases, small proteins (defensins and thionins) and lipid transfer proteins
410 (LTPs) [64,65]. Most of the PR-proteins were down-regulated in our study, suggesting that
411 drought stress posed negative effect on PR-proteins defense response. Contrarily, most of
412 the genes related to dirigent-proteins (DIR), play role in lignin formation and proline-
413 related proteins were up-regulated, suggesting their possible defensive-role in grapevine in
414 response to drought stress.

415 **Conclusion**

416 Our results have provided substantial evidences to demonstrate that grapevine adaptation to
417 drought stress is a multistep component system consisting of several genes that regulates various
418 pathways. Out of 12,451 DEGs, 7987 DEGs were up-regulated and 4,464 DEGs were down-
419 regulated. Nearly 2 fold up-regulations of DEGs clearly indicate their defense role in grapevine
420 under various pathways in response to drought stress. The significant increase in the activity of
421 ROS enzymes and hormones level revealed the defensive role of these enzymes and hormones
422 during drought stress in grapevine leaves. Overall, study concludes that drought has severe
423 effects on growth and physiology of the grapevine, but defense-related pathways assist grapevine
424 to mitigate the drought severity.

Materials and Methods

Plant material and drought treatments

Two-year old grapevine (*V. vinifera* cv. 'Summer Black') pot grown plants were selected as experimental material which were grown in standard greenhouse condition ($25 \pm 5°C$) under 16-h light/8-h dark photoperiod and 65% relative humidity (RH) at the Nanjing Agricultural University-Nanjing, China. Grapevine plants were subjected to drought with an interval of 20 days against control, each with three biological replicates. The fourth unfolded leaf from the shoot apex was collected from the each replicates of both control and drought treatment with the interval of 0 and 20th day, respectively, and the three samples were mixed to make one composite sample. After harvesting, the samples were immediately put in liquid nitrogen and then stored at $-80°C$ until analysis.

Determination of important biochemistry and physiology-related traits

The chlorophyll a and b contents was determined using spectrophotometer at 663 and 645 nm, respectively as briefly explained by Leng et al. (2015). Photosynthesis activity, stomatal conductance and CO_2 assimilation rate were carried out on mature leaf between 4th to 7th nodes from the shoot base for both control and drought treatment; between 9:00 - 11:00 AM measured using LI-COR (LI-6400XT, Germany) meter as described by Tombesi et al. (2015). Malondialdehyde (MDA) contents were quantified by using thiobartiburic acid. The activities of antioxidant enzymes (SOD, POD and CAT) were measured using the method briefly described by Haider, et al. [66]. The activities of indole-acetic acid (IAA), abscisic acid (ABA), jasmonic acid (JA), gibberellic acid (GA) and brassinosteroid (BR) were measured following the method of Tombesi, et al. [27]. Three technical repeats were generated for all the quantifications. Data was subjected to one-way analysis of variance (ANOVA) at $p < 0.05$, using MINITAB (ver. 16) and represented as mean $\pm$ standard deviation (SD).

RNA extraction, cDNA library construction and Illumina deep sequencing

Total RNA from leaf samples of both control and drought-stressed were extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) (1% agarose gel buffered by Tris–acetate-EDTA was run to indicate the integrity of the RNA.) and subsequently used for mRNA purification and library construction with the Ultra™ RNA Library Prep Kit for Illumina (NEB, USA) following the manufacturer's instructions. The samples were sequenced on an Illumina HiseqTM2500 for 48h.

Analysis of gene expression level, gene ontology (GO) and Kyoto encyclopedia of genes and genomics (KEGG)

After adaptor trimming and quality trimming, the clean reads were mapped to the *V. vinifera* transcriptome using Bowtie 1.1.2. Then, Sam tools and BamIndexStats.jar were used to calculate the gene expression level, and reads per kilobase per million (RPKM) value was computed from SAM files [67]. Gene expression differences between $\log_2$ and early stationary phase were obtained by MARS (MA-plot-based method with Random Sampling model), a package from DEGseq 3.3 (Leng et al., 2015). We simply defined genes with at least 2-fold change between two samples and FDR (false discovery rate) less

11

466   than 0.001 as differential expressed genes. Transcripts with $|log2FC| < 1$ were assumed to
467   have no change in their expression levels. The gene ontology (GO) enrichment (p-value <
468   0.05) was investigated by subjecting all DEGs to GO database
469   (http://www.geneontology.org/) in order to further classify genes or their products into
470   terms (molecular function, biological process and cellular component) helpful in
471   understanding genes biological functions. Kyoto encyclopedia of genes and genomics
472   (KEGG; the major public pathway-related database) was used to perform pathway
473   enrichment analysis of DEGs [68].

Illumina RNA-seq results validation by qRT-PCR

475   In order to validate the Illumina RNA-seq results the drought-stressed grapevine leaf
476   samples of each collection were applied to qRT-PCR analysis. Total RNA of the collected
477   samples was extracted following the above mentioned method, and then was reverse-
478   transcribed using the PrimeScript RT Reagent Kit with gDNA Eraser (Takara, Dalian,
479   China), following the manufacturers' protocol. Gene specific qRT-PCR primers were
480   designed using Primer3 software (http://primer3.ut.ee/), for 20 selected genes with the
481   sequence data in 3'UTR (Table S12). qRT-PCR was carried out using an ABI PRISM
482   7500 real-time PCR system (Applied Biosystems, USA). Each reaction contains 10μl
483   2×SYBR Green Master Mix Reagent (Applied Biosystems, USA), 2.0μl cDNA sample,
484   and 400 nM of gene-specific primer in a final volume of 20μl. PCR conditions were 2 min
485   at 95°C, followed by 40 cycles of heating at 95°C for 10s and annealing at 60°C for 40s. A
486   template-free control for each primer pair was set for each cycle. All PCR reactions were
487   normalized using the Ct value corresponding to the Grapevine UBI gene. Three biological
488   replicates were generated and three measurements were performed on each replicate.

Acknowledgments

Conflict of Interest statement

495   The authors declare that the research was conducted in the absence of any commercial or
496   financial relationships that could be construed as a potential conflict of interest.

Authors' contributions

498   Conceived and designed the experiments: MSH, JF. Perform the experiment: MSH, CZ.
499   Analyzed the data: MSH, TP, LS. Contributed in reagents/ materials/ analysis tools: MSH,
500   MMK, SJ, LA. Manuscript writing: MSH, MMK, JF. All the authors approved the final draft of
501   manuscript.

Abbreviations

503   ABA: Abscisic acid; ANOVA: Analysis of variance; BR: Brassinosteroid; CAT: Catalase;
504   DEGs: Differentially-expressed genes; ESTs: Expressed sequence tags; FDR: False discovery
505   rate; GA: Gibberellic acid; IAA: Indole-acetic acid; JA: Jasmonic acid; MARS: MA-plot-based
506   method with Random Sampling model; MDA: Malondialdehyde; POD: peroxidase; qRT-PCR:

507 quantitative real-time PCR; RNA-seq: RNA-sequencing; ROS: Reactive oxygen species; SD:
508 Standard deviation; SOD: Superoxide dismutase.

References

510 1   Griesser, M. *et al.* Severe drought stress is affecting selected primary metabolites,
511   polyphenols, and volatile metabolites in grapevine leaves (Vitis vinifera cv. Pinot noir).
512   *Plant Physiology and Biochemistry* **88**, 17-26 (2015).
513 2   Ma, Y., Qin, F. & Tran, L. S. Contribution of genomics to gene discovery in plant abiotic
514   stress responses. *Mol Plant* **5**, 1176-1178, doi:10.1093/mp/sss085 (2012).
515 3   Krasensky, J. & Jonak, C. Drought, salt, and temperature stress-induced metabolic
516   rearrangements and regulatory networks. *Journal of experimental botany* **63**, 1593-1608
517   (2012).
518 4   Ashraf, M. & Foolad, M. Roles of glycine betaine and proline in improving plant abiotic
519   stress resistance. *Environmental and Experimental Botany* **59**, 206-216 (2007).
520 5   Khan, M. S. The role of DREB transcription factors in abiotic stress tolerance of plants.
521   *Biotechnology & Biotechnological Equipment* **25**, 2433-2442 (2011).
522 6   Vinocur, B. & Altman, A. Recent advances in engineering plant tolerance to abiotic
523   stress: achievements and limitations. *Current opinion in biotechnology* **16**, 123-132
524   (2005).
525 7   Shinozaki, K., Yamaguchi-Shinozaki, K. & Seki, M. Regulatory network of gene
526   expression in the drought and cold stress responses. *Current opinion in plant biology* **6**,
527   410-417 (2003).
528 8   Valliyodan, B. & Nguyen, H. T. Understanding regulatory networks and engineering for
529   enhanced drought tolerance in plants. *Current opinion in plant biology* **9**, 189-195
530   (2006).
531 9   Chaves, M. M. *et al.* Deficit irrigation in grapevine improves water-use efficiency while
532   controlling vigour and production quality. *Annals of Applied Biology* **150**, 237-252
533   (2007).
534 10   Escalona, J. M. *et al.* Responses of leaf night transpiration to drought stress in Vitis
535   vinifera L. *Agricultural water management* **118**, 50-58 (2013).
536 11   Schultz, H. Climate change and viticulture: a European perspective on climatology,
537   carbon dioxide and UV-B effects. *Australian Journal of grape and wine research* **6**, 2-12
538   (2000).
539 12   Lovisolo, C., Schubert, A. & Sorce, C. Are xylem radial development and hydraulic
540   conductivity in downwardly-growing grapevine shoots influenced by perturbed auxin
541   metabolism? *New Phytologist* **156**, 65-74 (2002).
542 13   Soar, C. J. *et al.* Grape vine varieties Shiraz and Grenache differ in their stomatal
543   response to VPD: apparent links with ABA physiology and gene expression in leaf tissue.
544   *Australian journal of grape and wine research* **12**, 2-12 (2006).
545 14   Vandeleur, R. K. *et al.* The role of plasma membrane intrinsic protein aquaporins in
546   water transport through roots: diurnal and drought stress responses reveal different
547   strategies between isohydric and anisohydric cultivars of grapevine. *Plant Physiology*
548   **149**, 445-460 (2009).

549   15   Silva, P. & Gerós, H. Regulation by salt of vacuolar H+-ATPase and H+-
550   pyrophosphatase activities and Na+/H+ exchange. *Plant signaling & behavior* **4**, 718-726
551   (2009).
552   16   Wang, C. *et al.* Depiction of Grapevine Phenology by Gene Expression Information and a
553   Test of its Workability in Guiding Fertilization. *Plant molecular biology reporter* **32**,
554   1070-1084 (2014).
555   17   Mafakheri, A., Siosemardeh, A., Bahramnejad, B., Struik, P. & Sohrabi, Y. Effect of
556   drought stress on yield, proline and chlorophyll contents in three chickpea cultivars.
557   *Australian journal of crop science* **4**, 580 (2010).
558   18   Homayoun, H., Daliri, M. S. & Mehrabi, P. Effect of drought stress on leaf chlorophyll in
559   corn cultivars (Zea mays). *Middle-East J Sci Res* **9**, 418-420 (2011).
560   19   Hörtensteiner, S. & Kräutler, B. Chlorophyll breakdown in higher plants. *Biochimica et
561   Biophysica Acta (BBA)-Bioenergetics* **1807**, 977-988 (2011).
562   20   Sakuraba, Y., Tanaka, R., Yamasato, A. & Tanaka, A. Determination of a chloroplast
563   degron in the regulatory domain of chlorophyllide a oxygenase. *Journal of Biological
564   Chemistry* **284**, 36689-36699 (2009).
565   21   Scheumann, V., Schoch, S. & Rüdiger, W. Chlorophyll b reduction during senescence of
566   barley seedlings. *Planta* **209**, 364-370 (1999).
567   22   Barry, C. S. The stay-green revolution: Recent progress in deciphering the mechanisms
568   of chlorophyll degradation in higher plants. *Plant Science* **176**, 325-333 (2009).
569   23   Harpaz-Saad, S. *et al.* Chlorohyllase Is a Rate-Limiting Enzyme in Chlorophyll
570   Catabolism and Is Posttranslationally Regulated. *The Plant Cell*, 1007-1022 (2007).
571   24   Büchert, A. M. Chlorophyllase versus pheophytinase as candidates for chlorophyll
572   dephytilation during senescence of broccoli. *Journal of plant physiology* **168**, 337-343
573   (2011).
574   25   Du, L. *et al.* Characterization of the stage dependency of high temperature on green
575   ripening reveals a distinct chlorophyll degradation regulation in banana fruit. *Scientia
576   Horticulturae* **180**, 139-146 (2014).
577   26   Leng, X. *et al.* Comparative transcriptome analysis of grapevine in response to copper
578   stress. *Scientific reports* **5** (2015).
579   27   Tombesi, S. *et al.* Stomatal closure is induced by hydraulic signals and maintained by
580   ABA in drought-stressed grapevine. *Scientific reports* **5** (2015).
581   28   Nelson, N. & Ben-Shem, A. Photosystem I reaction center: past and future.
582   *Photosynthesis research* **73**, 193-206 (2002).
583   29   Pinheiro, C. & Chaves, M. Photosynthesis and drought: can we make metabolic
584   connections from available data? *Journal of experimental botany* **62**, 869-882 (2011).
585   30   Osakabe, Y., Osakabe, K., Shinozaki, K. & Tran, L.-S. P. Response of plants to water
586   stress. *Front. Plant Sci* **5**, 10.3389 (2014).
587   31   Zivcak, M. *et al.* Photosynthetic electron transport and specific photoprotective responses
588   in wheat leaves under drought stress. *Photosynthesis research* **117**, 529-546 (2013).
589   32   HongBo, S., ZongSuo, L. & MingAn, S. Changes of anti-oxidative enzymes and MDA
590   content under soil water deficits among 10 wheat (Triticum aestivum L.) genotypes at
591   maturation stage. *Colloids and Surfaces B: Biointerfaces* **45**, 7-13 (2005).
592   33   Yang, S. & Deng, X. Effects of drought stress on antioxidant enzymes in seedlings of
593   different wheat genotypes. *Pak. J. Bot* **47**, 49-56 (2015).

594 34   Abedi, T. & Pakniyat, H. Antioxidant enzyme changes in response to drought stress in
595   ten cultivars of oilseed rape (Brassica napus L.). *Czech J. Genet. Plant Breed* **46**, 27-34
596   (2010).
597 35   Sofo, A., Dichio, B., Xiloyannis, C. & Masia, A. Antioxidant defences in olive trees
598   during drought stress: changes in activity of some antioxidant enzymes. *Functional Plant*
599   *Biology* **32**, 45-53 (2005).
600 36   Apel, K. & Hirt, H. Reactive oxygen species: metabolism, oxidative stress, and signal
601   transduction. *Annu. Rev. Plant Biol.* **55**, 373-399 (2004).
602 37   Bolwell, G. P. Role of active oxygen species and NO in plant defence responses. *Current*
603   *opinion in plant biology* **2**, 287-294 (1999).
604 38   Chaves, M., Flexas, J. & Pinheiro, C. Photosynthesis under drought and salt stress:
605   regulation mechanisms from whole plant to cell. *Annals of botany* **103**, 551-560 (2009).
606 39   Ioannidi, E. *et al.* Expression profiling of ascorbic acid-related genes during tomato fruit
607   development and ripening and in response to stress conditions. *Journal of Experimental*
608   *Botany*, ern322 (2009).
609 40   Yamaguchi-Shinozaki, K. & Shinozaki, K. Transcriptional regulatory networks in
610   cellular responses and tolerance to dehydration and cold stresses. *Annu. Rev. Plant Biol.*
611   **57**, 781-803 (2006).
612 41   Fujii, H., Verslues, P. E. & Zhu, J.-K. Arabidopsis decuple mutant reveals the importance
613   of SnRK2 kinases in osmotic stress responses in vivo. *Proceedings of the National*
614   *Academy of Sciences* **108**, 1717-1722 (2011).
615 42   Yoshida, R. *et al.* ABA-activated SnRK2 protein kinase is required for dehydration stress
616   signaling in Arabidopsis. *Plant and Cell Physiology* **43**, 1473-1483 (2002).
617 43   Chan, Z. Expression profiling of ABA pathway transcripts indicates crosstalk between
618   abiotic and biotic stress responses in Arabidopsis. *Genomics* **100**, 110-115 (2012).
619 44   Achard, P., Renou, J.-P., Berthomé, R., Harberd, N. P. & Genschik, P. Plant DELLAs
620   restrain growth and promote survival of adversity by reducing the levels of reactive
621   oxygen species. *Current Biology* **18**, 656-660 (2008).
622 45   Zhang, X., Wang, C., Zhang, Y., Sun, Y. & Mou, Z. The Arabidopsis mediator complex
623   subunit16 positively regulates salicylate-mediated systemic acquired resistance and
624   jasmonate/ethylene-induced defense pathways. *The Plant Cell* **24**, 4294-4309 (2012).
625 46   Seo, J. S. *et al.* OsbHLH148, a basic helix-loop-helix protein, interacts with OsJAZ
626   proteins in a jasmonate signaling pathway leading to drought tolerance in rice. *The Plant*
627   *Journal* **65**, 907-921 (2011).
628 47   Wilkinson, S. & Davies, W. J. ABA-based chemical signalling: the co-ordination of
629   responses to stress in plants. *Plant, cell & environment* **25**, 195-210 (2002).
630 48   Wilkinson, S. & Davies, W. J. Manipulation of the apoplastic pH of intact plants mimics
631   stomatal and growth responses to water availability and microclimatic variation. *Journal*
632   *of Experimental Botany* **59**, 619-631 (2008).
633 49   Schachtman, D. P. & Goodger, J. Q. Chemical root to shoot signaling under drought.
634   *Trends in plant science* **13**, 281-287 (2008).
635 50   Feng, Y., Yin, Y. & Fei, S. Down-regulation of BdBRI1, a putative brassinosteroid
636   receptor gene produces a dwarf phenotype with enhanced drought tolerance in
637   Brachypodium distachyon. *Plant Science* **234**, 163-173 (2015).

638   51   Kishor, P. K. *et al.* Regulation of proline biosynthesis, degradation, uptake and transport
639   in higher plants: its implications in plant growth and abiotic stress tolerance. *Curr Sci* **88**,
640   424-438 (2005).

641   52   Lea, P. J. M. *Intermediary nitrogen metabolism.* (1990).

642   53   Kiyosue, T., Yoshiba, Y., Yamaguchi-Shinozaki, K. & Shinozaki, K. A nuclear gene
643   encoding mitochondrial proline dehydrogenase, an enzyme involved in proline
644   metabolism, is upregulated by proline but downregulated by dehydration in Arabidopsis.
645   *The Plant Cell* **8**, 1323-1335 (1996).

646   54   Peng, Z., Lu, Q. & Verma, D. Reciprocal regulation of Δ 1-pyrroline-5-carboxylate
647   synthetase and proline dehydrogenase genes controls proline levels during and after
648   osmotic stress in plants. *Molecular and General Genetics MGG* **253**, 334-341 (1996).

649   55   Akula, R. & Ravishankar, G. A. Influence of abiotic stress signals on secondary
650   metabolites in plants. *Plant signaling & behavior* **6**, 1720-1731 (2011).

651   56   Maeda, H. & Dudareva, N. The shikimate pathway and aromatic amino acid biosynthesis
652   in plants. *Annual review of plant biology* **63**, 73-105 (2012).

653   57   Tanaka, Y. *et al.* Structure and characterization of a cDNA clone for phenylalanine
654   ammonia-lyase from cut-injured roots of sweet potato. *Plant physiology* **90**, 1403-1407
655   (1989).

656   58   Richard, S., Lapointe, G., Rutledge, R. G. & Séguin, A. Induction of chalcone synthase
657   expression in white spruce by wounding and jasmonate. *Plant and Cell Physiology* **41**,
658   982-987 (2000).

659   59   Winkel-Shirley, B. Flavonoid biosynthesis. A colorful model for genetics, biochemistry,
660   cell biology, and biotechnology. *Plant physiology* **126**, 485-493 (2001).

661   60   Jonnalagadda, V., Toth, K. & Richard, J. P. Isopentenyl diphosphate isomerase catalyzed
662   reactions in D2O: product release limits the rate of this sluggish enzyme-catalyzed
663   reaction. *Journal of the American Chemical Society* **134**, 6568-6570 (2012).

664   61   Tholl, D. Terpene synthases and the regulation, diversity and biological roles of terpene
665   metabolism. *Current opinion in plant biology* **9**, 297-304 (2006).

666   62   Li, J., He, Q., Sun, H. & Liu, X. Acclimation-dependent expression of heat shock protein
667   70 in Pacific abalone (Haliotis discus hannai Ino) and its acute response to thermal
668   exposure. *Chinese Journal of Oceanology and Limnology* **30**, 146-151 (2012).

669   63   Nollen, E. A. & Morimoto, R. I. Chaperoning signaling pathways: molecular chaperones
670   as stress-sensingheat shock'proteins. *Journal of cell science* **115**, 2809-2816 (2002).

671   64   Van Loon, L. Pathogenesis-related proteins. *Plant Molecular Biology* **4**, 111-116 (1985).

672   65   Van Loon, L. & Van Strien, E. The families of pathogenesis-related proteins, their
673   activities, and comparative analysis of PR-1 type proteins. *Physiological and molecular*
674   *plant pathology* **55**, 85-97 (1999).

675   66   Haider, M. S. *et al.* Fruit developmental stages effects on biochemical attributes in date
676   palm. *Pak. J. Agri. Sci* **50**, 577-583 (2013).

677   67   Pervaiz, T. *et al.* Transcriptomic Analysis of Grapevine (cv. Summer Black) Leaf, Using
678   the Illumina Platform. *PloS one* **11**, e0147369 (2016).

679   68   Kanehisa, M. *et al.* KEGG for linking genomes to life and the environment. *Nucleic acids*
680   *research* **36**, D480-D484 (2008).

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682

683 **Table 1.** Comparison of some physiological and biochemical parameters in grapevine leaves
684 under drought stress environment

Physiological and biochemical parameters	Control	Drought treatment	Range of increasing %
Chlorophyll contents (mg g^{-1})	1.16 ± 0.08	0.85 ± 0.09	-26.72
Chla contents (mg g^{-1})	0.43 ± 0.11	0.28 ± 0.06	-34.88
Chlb contents (mg g^{-1})	0.73 ± 0.06	0.57 ± 0.04	-21.92
Photosynthesis activity (μmole m^{-2}sec^{-1})	23.67 ± 0.81	16.08 ± 0.75	-32.20
Stomatal conductance (μmole m^{-2}sec^{-1})	0.15 ± 0.03	0.09 ± 0.02	40.00
Net CO2 assimilation (μmole m^{-2}sec^{-1})	9 ± 0.03	5 ± 0.03	44.44
MDA contents (nmol/g)	5.35 ± 0.21	8.61 ± 0.25	60.93
SOD activity (U/ g/ min)	371.56 ± 10.21	650.85 ± 15.7	75.16
POD activity (U/ g/ min)	18.23 ± 0.97	43.9 ± 1.01	140.81
CAT activity (U/ g/ min)	6.32 ± 1.21	19.01 ± 0.99	200.79
Proline (ng/g FW)	1.124 ± 0.04	1.711 ± 0.05	52.37

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686

687 **Table 2.** List of differentially-expressed genes related to chlorophyll degradation and photosynthesis in
688 grapevine perceived during drought stress.

Trait name	Description	No. of up-regulated	No. of down-regulated	sum
Chlorophyll Metabolism	Chlorophyll a synthesis	9	6	15
	Chlorophyll cycle	1	1	1
	Chlorophyll degradation	3	3	6
Photosystem II	psbB	0	2	2
	psbC	0	2	2
	psbW	0	1	1
Photosystem I	psaB	0	2	2
Cytochrome b6-f complex	petA	0	1	1
	petC	1	2	3
Phtosynthetic electron transport	petF	0	2	2
	petH	2	0	2
F-type ATPase	ATPF1B	0	1	1
	ATPF1A	0	1	1
	ATPF1G	0	1	1
	ATPF0C	0	1	1
Photosynthesis-antenna proteins	LHCB1	0	1	1
	LHCB2	0	1	1
	LHCB3	0	1	1
	LHCB6	0	1	

689 psbB, Photosystem II CP47 chlorophyll apoprotein gene; psbC, Photosystem II CP43 chlorophyll
690 apoprotein gene; psbW, Photosystem II reaction center W protein; psaB, photosystem I P700 apoprotein
691 A2 gene; petA, cytochrome f; petC, cytochrome b6-f complex iron-sulfur subunit 1; petF, ferredoxin-3;
692 petH, ferredoxin--NADP reductase, leaf-type isozyme; ATPF1B, ATP synthase CF1 beta; ATPF1A, ATP
693 synthase CF1 alpha; ATPF1G, ATP synthase gamma; LHCB1, chlorophyll a-b binding protein of LHCII;
694 LHCB2, light harvesting chlorophyll A/B binding protein; LHCB3, light-harvesting chlorophyll binding
695 protein 3 gene; LHCB6, chlorophyll a-b binding protein CP24 10A.

696 **Table 3.** List of differentially-expressed genes related to chlorophyll degradation and photosynthesis in
697 grapevine perceived during drought stress.

Trait name	Description	No. of up-regulated	No. of down-regulated	sum
ROS synthesis	Rboh	1	1	2
	AO	5	0	5
	Fe-SOD	2	0	2
ROS scavenging	POD	2	4	6
	CAT	3	0	3
	MDAR	1	0	1
GSH-AsA cycle	DHAR	1	0	1
	GR	1	0	1
	Grx	2	4	6
GPX pathway	GPX	1	0	1
	GST	22	4	26
Prx/Trx	Prx	0	1	1
	Trx	5	2	7
Cyanide-resistant respiration	AOX	2	1	3
Copper-containing enzymes	PPO	0	2	2

698 Rboh, respiratory burst oxidase; AO, amine oxidase; Fe-SOD, Fe superoxide dismutase; POD,
699 peroxidase; CAT, catalase, APX, ascorbate peroxidase; MDAR, monodehydroascorbate reductase;
700 DHAR, dehydroascorbate reductase; GR, glutathione reductase; Grx, glutaredoxin; GPX, glutathione
701 peroxidase; GST, glutathione S transferase; Prx, peroxiredoxin; Trx, thioredoxin; AOX, alternative
702 oxidase, PPO, polyphenol oxidase.

Table 4. Elucidation on differential expression of genes related to secondary metabolites under drought stress.

Trait name	Description	No. of up-regulated	No. of down-regulated	sum
	DAHPS3	1	0	1
	B3D/SDH	0	2	2
	SHK	0	1	1
Shikimate acid pathway	CS1	1	0	1
	AnPRT	0	2	2
	IGPS	1	0	1
	TS	1	0	1
Alkaloids biosynthetic pathway	STR3	0	1	1
	DAT	0	1	1
	PAL	4	4	8
	TC4M	0	1	1
	STS	6	7	13
	4CL	1	1	2
Anthocyanin biosynthetic pathway	F3D	1	0	1
	FLSI	1	2	3
	DFR	0	2	2
	UFGT	0	1	1
	ANR	1	0	1
	SOH	1	0	1
	CA3M	0	2	2
	COM	2	0	0
Lignin biosynthetic pathway	CCR1	1	1	2
	CAD1	1	0	1
	POD	2	5	7
	LAC	1	3	4
	HMGS	1	1	2
	DXPS	1	1	2
Terpenoid biosynthetic pathway	IPI2	1	0	1
	TSE	1	0	1
	SED	0	2	2

DAHPS3, 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase 03; B3D/SDH, bifunctional 3-dehydroquinate dehydratase/shikimate dehydrogenase; SHK, shikimate kinase; CS1, chorismate synthase 1; AnPRT, anthranilate phosphoribosyltransferase; IGPS, indole-3-glycerol phosphate synthase; TS, tryptophan synthase beta chain 1; STR3; strictosidine synthase 3; DAT, D-amino-acid transaminase; PAL, phenylalanine ammonia-lyase; TC4M, Trans-cinnamate 4-monooxygenase; STS, stilbene synthase; 4CL, 4-coumarate--CoA ligase; F3D, flavanone 3-dioxygenase; FLS1, flavonol synthase/flavanone 3-hydroxylase; DFR, dihydroflavonol-4-reductase; UFGT; anthocyanidin 3-O-glucosyltransferase 2; ANR, anthocyanidin reductase; SOH, shikimate O-hydroxycinnamoyltransferase; CA3M, caffeic acid 3-O-methyltransferase; COM, caffeoyl-CoA O-methyltransferase; CCR1, cinnamoyl-CoA reductase 1; CAD1; cinnamyl alcohol dehydrogenase 1; POD; Peroxidase; LAC; laccase; HMGS, hydroxymethylglutaryl-CoA synthase; DXPS, 1-deoxy-D-xylulose-5-phosphate synthase; IPI2, isopentenyl diphosphate isomerase II; TSE, terpene synthase; SED, squalene epoxidase.

719 **Table 5.** List of differentially-expressed genes related to heat-shock proteins (HSPs) and pathogens
720 resistance (PRs) proteins in grapevine perceived during drought stress.

Trait name	Description	No. of up-regulated	No. of down-regulated	sum
	HSP101	0	1	1
	HSP90	1	2	3
	HSP70	1	1	2
Heat shock proteins	small HSP	12	6	18
	other HSP	17	3	20
	heat-stress transcription factor	4	1	5
PR-1	pathogenesis-related protein 2	4	6	10
PR-2	Beta-1,3-glucanase	4	5	9
PR-3,4,8,11	chitinase	4	15	19
PR-5	Thaumatin-like protein	6	8	14
PR-10	Pathogenesis-related protein 10	2	2	4
PR-14	lipid transfer protein	5	5	10
PR-15	germin-like protein 2	2	2	4
PTI	transcriptional activator		2	2
dirigent protein		3	7	10
proline related protein		6	6	12

721

Figureure legends

Figure 1. Chlorophyll metabolic pathway in drought-stress grapevine leaves. GLTL, Glutamate tRNA ligase; HemA, Glutamate tRNA reductase 1; GSA, Glutamate-1-semialdehyde; ALAD, Delta-aminolevulinic acid dehydrates; PBGD, porphobilinogen deaminase; UROS, Uroporphyrinogen III synthase; RMA1, Radical S-adenosyl methionine domain-containing protein 1; PPOX1; Proporphynogen oxidase 1; PPOX, Proporphynogen oxidase; UROD, Uroporphyrinogen III decarboxylase; CHLH, Magnesium chelatase H subunit; CHL1, Magnesium-chelatase I subunit; CHLD, Magnesium chelatase D subunit; CHLM, Mg-proto IX methyltransferase; CRD1, Mg-protophyrin IX monomethylester (oxidative) cyclase; POR, Protochlorophyllide oxidoreductase; DHR, Dehydrogenase/reductase SDR family member; SCD, Short chain dehydrogenase, TIC32; CHLG, CAO; Chlorophyllide a oxygenase; CBR, Chlorophyll(ide) b reductase NYC1; CLH1, Chlorophyllase-I; CLH2, Chlorophyllase-II; PAO, Pheophorbide a oxygenase; PDT, Protochlorophyllide-dependent translocon component 52.

Figure 2. Reactive oxygen species (ROS) scavenging pathway in plants. (a) The ascorbate-glutathione (AsA-GSH) cycle, (b) The glutathione peroxidase (GPX) cycle. SOD (superoxide dismutase) initiate the line of defense by converting O_2^- into H_2O_2, ehich is further detoxified by CAT (catalses), APX (ascorbate peroxidases (APX) and GPX (glutathione ascorbate).

Abbreviations: DHA, dehydroascorbate; GSH, glutathione; GSSG, oxidized glutathione; GR, gkutathione reductase; MDAR, monodehydroascorbate reductase; DHAR, dehydroascorbate reductase.
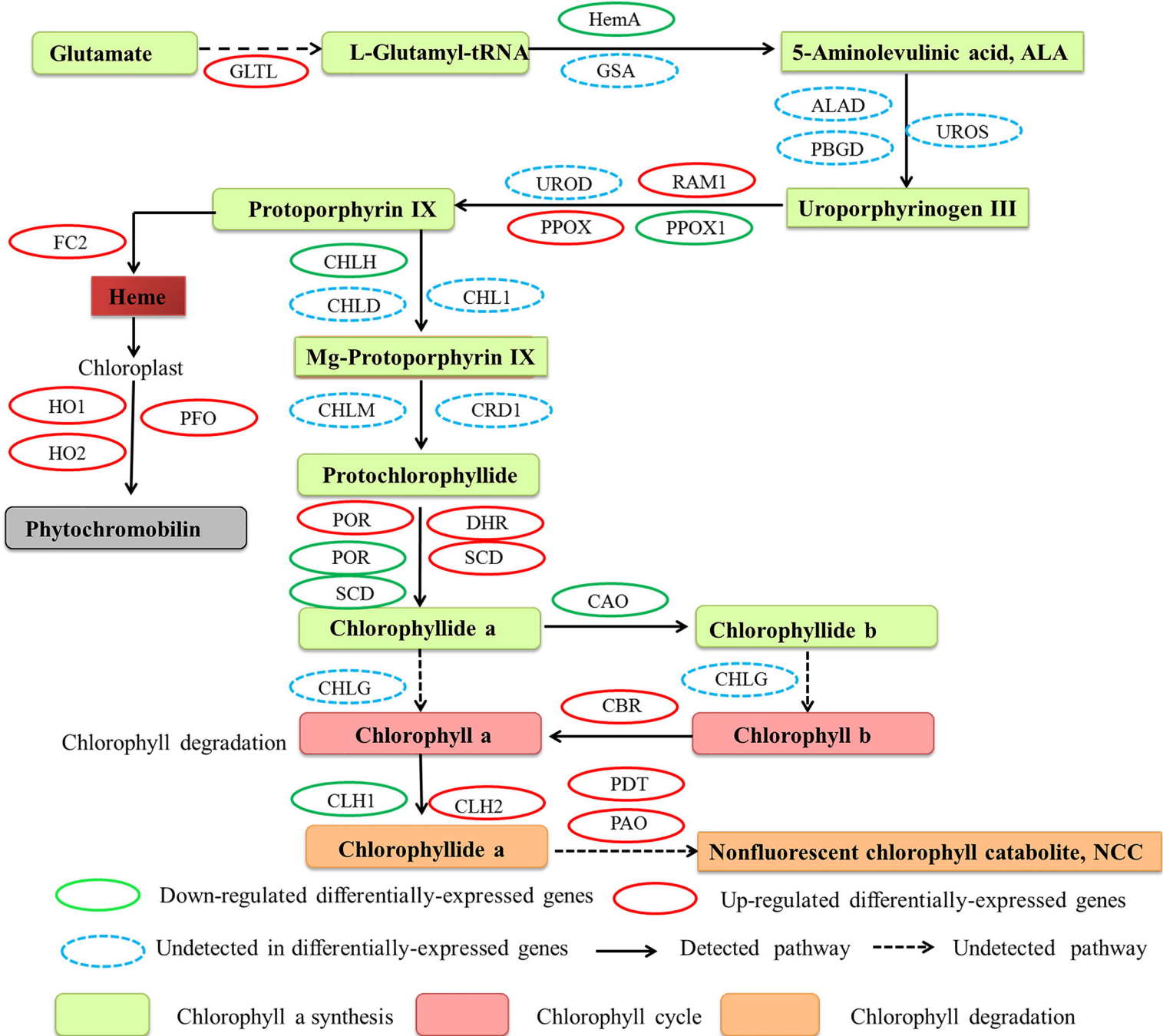
Figure 3. The activities of different hormone, including IAA (indole-acetic acid), ABA (abscisic acid), JA (jasmonic acid), GA (gibberellic acid) and BR (brassinsteroid) in control and drought treatment.

Figure 4. Differential expressions of genes during biosynthesis and degradation of proline in response to drought stress. Given numbers represents the individual genes catalyzing specific reactions. P5CS, pyroline-5-carboxylate synthetase; ARG, arginase; δ-AOT; ornithine-δ-aminotransferase; P5CR, pyrroline 5-carboxylate reductase; PDH, Proline dehydrogenase; P5CDH, Pyrroline-5-carboxyate dehydrogenase.
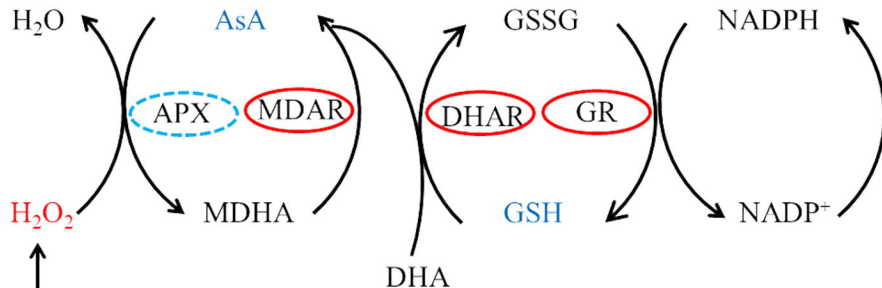
Figure 5. Differential expression of genes related to secondary metabolites under drought stress. DAHPS3, 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase 03; B3D/SDH, bifunctional 3-dehydroquinate dehydratase/shikimate dehydrogenase; SHK, shikimate kinase; CS1, chorismate synthase 1; AnPRT, anthranilate phosphoribosyltransferase; IGPS, indole-3-glycerol phosphate synthase; TS, tryptophan synthase beta chain 1; STR3; strictosidine synthase 3; DAT, D-amino-acid transaminase; PAL, phenylalanine ammonia-lyase; TC4M, Trans-cinnamate 4-monooxygenase; STS, stilbene synthase; 4CL, 4-coumarate--CoA ligase; F3D, flavanone 3-dioxygenase; FLS1, flavonol synthase/flavanone 3-hydroxylase; DFR, dihydroflavonol-4-reductase; UFGT; anthocyanidin 3-O-glucosyltransferase 2; ANR, anthocyanidin reductase; SOH, shikimate O-hydroxycinnamoyltransferase; CA3M, caffeic acid 3-O-methyltransferase; COM, caffeoyl-CoA O-methyltransferase; CCR1, cinnamoyl-CoA reductase 1; CAD1; cinnamyl alcohol dehydrogenase 1; POD; Peroxidase; LAC; laccase.

Figure 6. Verification of relative expression levels of DEGs by qRT-PCR. Error bars indicate standard deviation from 3 technical replicates of RT-qPCR. Expression patterns of 16 DEGs selected from different elucidated pathways by qRT-PCR (blue bar) and RNA-Seq (red dot). (1) Seq ID: VIT_07s0151g00110.t01 (Chlorophyllase-1), (2) Seq ID: VIT_00s0396g00010.t01 (psbC; Photosystem II CP43 chlorophyll apoprotein gene), (3) Seq ID: VIT_00s2608g00020.t01 (psbB; Photosystem II CP47 chlorophyll apoprotein gene), (4) Seq ID: VIT_18s0001g08620.t01 (psaB; photosystem I P700 apoprotein A2 gene), (5) Seq ID: VIT_19s0027g01930.t01 (peroxiredoxin (Prx), (6) Seq ID:
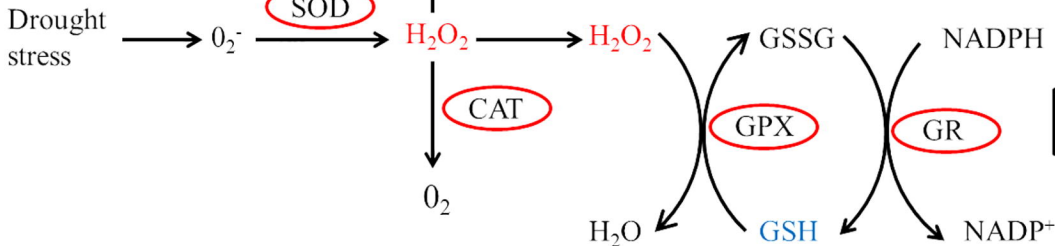
22

766   VIT_11s0037g00940.t01   (S-adenosylmethionine   decarboxylase   proenzyme),   (7)   Seq   ID:
767   VIT_12s0055g01020.t01   (peroxidase N1-like), (8) Seq ID: VIT_18s0001g08550.t01 (squalene
768   monooxygenase), (9) Seq ID: VIT_06s0061g00790.t01 (Pheophorbide a oxygenase), (10) Seq ID:
769   VIT_17s0000g06130.t01 (glutathione S-transferase U9), (11) Seq ID: VIT_04s0023g03230.t01 (auxin-
770   induced protein 15A-like), (12) Seq ID: VIT_01s0146g00350.t01 (BRASSINOSTEROID INSENSITIVE
771   1-associated receptor kinase 1), (13) Seq ID: VIT_01s0011g00480.t01 (glutamate 5-kinase), (14) Seq ID:
772   VIT_07s0129g00460.t01 (prolyl 4-hydroxylase 9), (15) Seq ID: VIT_16s0039g01360.t01 (phenylalanine
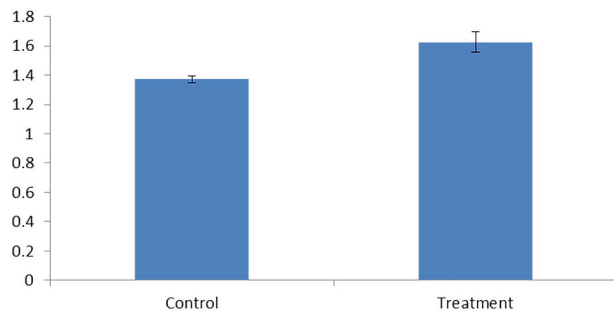773   ammonia-lyase), (16) Seq ID: VIT_03s0088g00710.t01 (pathogenesis-related protein PR-1).

Glutamate — GLTL → L-Glutamyl-tRNA — HemA / GSA → 5-Aminolevulinic acid, ALA

ALAD, PBGD, UROS → Uroporphyrinogen III

UROD, RAM1, PPOX, PPOX1 → Protoporphyrin IX

FC2 → Heme

Chloroplast

HO1, HO2, PFO → Phytochromobilin

CHLH, CHLD, CHL1 → Mg-Protoporphyrin IX

CHLM, CRD1 → Protochlorophyllide

POR, DHR, SCD → Chlorophyllide a — CAO → Chlorophyllide b

CHLG → Chlorophyll a — CBR ← Chlorophyll b — CHLG

Chlorophyll degradation

CLH1, CLH2 → Chlorophyllide a — PDT, PAO → Nonfluorescent chlorophyll catabolite, NCC

○ (green) Down-regulated differentially-expressed genes   ○ (red) Up-regulated differentially-expressed genes

○ (blue dashed) Undetected in differentially-expressed genes   → Detected pathway   --→ Undetected pathway

□ (green) Chlorophyll a synthesis   □ (pink) Chlorophyll cycle   □ (orange) Chlorophyll degradation
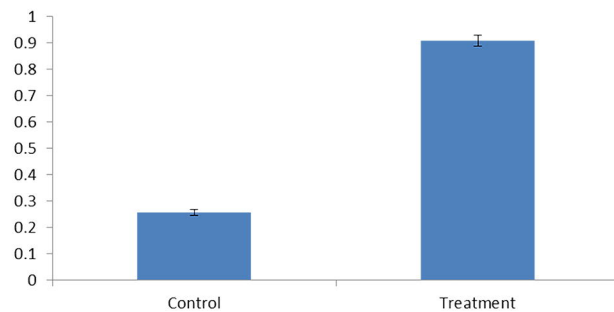
a. AsA-GSH cycle

b. GPX cycle

Up-regulated differentially-expressed genes
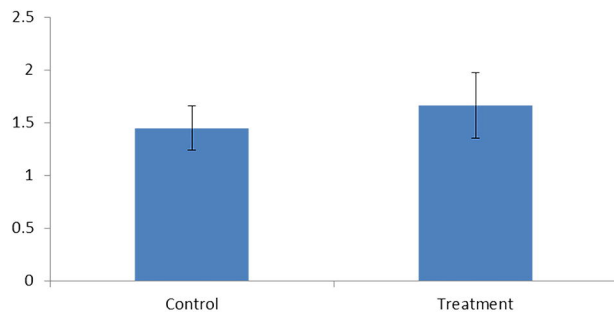
Not detected differentially-expressed genes

Ng/g FW

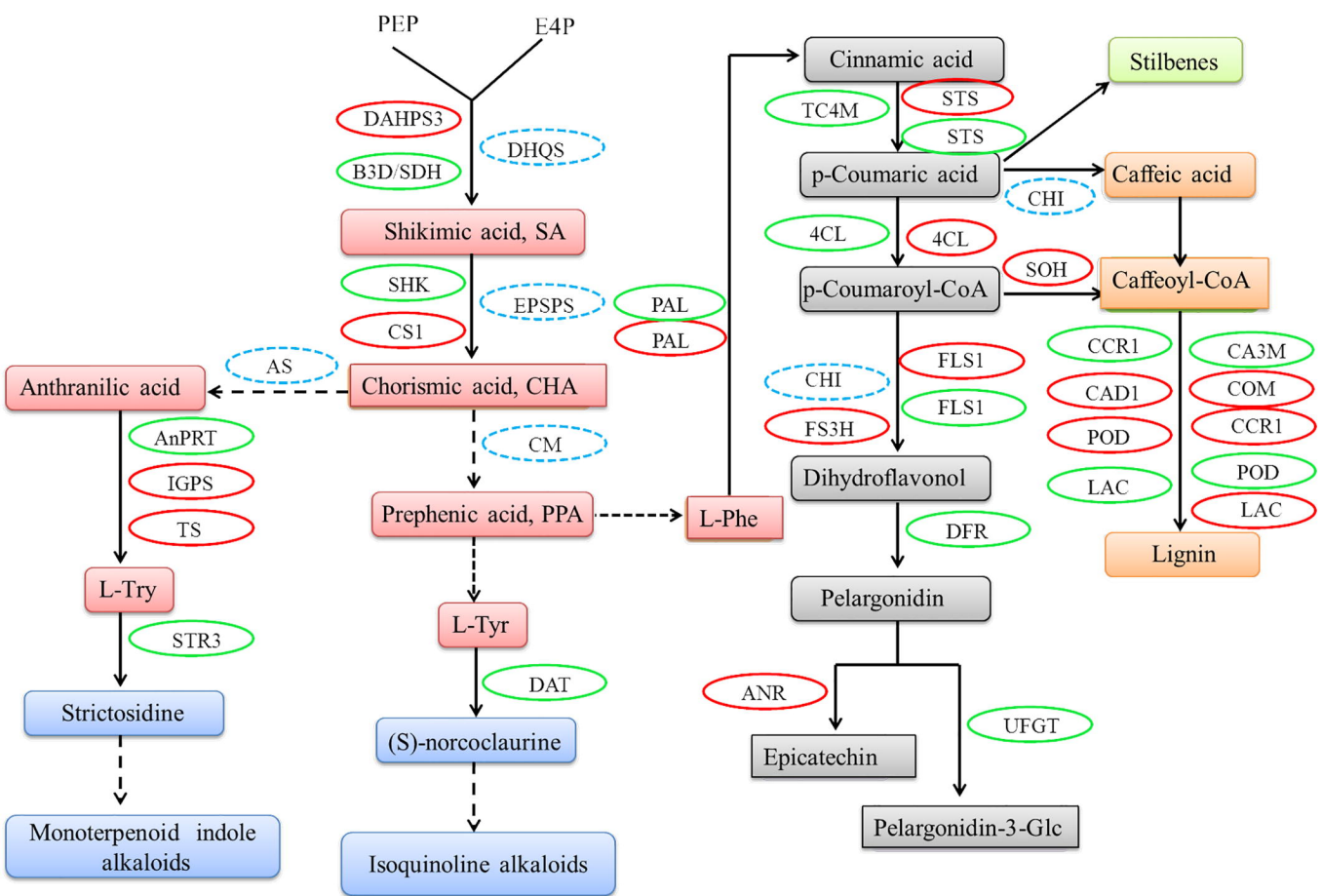Up-regulated differentially-expressed genes ⬭ (red solid oval)   Undetected in differentially-expressed genes ⬭ (blue dotted oval)

qRT-PCR
RNA-Seq

VIT_07s0151g00110.t01
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VIT_00s2608g00020.t01
VIT_18s0001g08620.t01

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VIT_11s0037g00940.t01
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VIT_01s0011g00480.t01
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Relative Expression

Length of drought treatment