Gene regulation mechanism in drought-responsive grapevine leaves as revealed by transcriptomic analysis

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Abstract

The transcriptome of drought-stressed grapevine leaves was analyzed using RNA-sequencing (RNA-seq) technology and compared. In this experiment, 12,451 differentially expressed genes (DEGs) were recorded in response to drought by RNA-seq, which includes 7,983 up-regulated and 4,468 down-regulated transcripts, proposing that drought stress has induced/repressed many genes. Biological and physiological analysis revealed that drought has influenced the chlorophyll metabolism and photosynthetic activity in grapevine leaves. Besides, various defensive mechanism-related pathways including, reactive oxygen species (ROS), hormonal signal transduction, proline metabolism, biosynthesis of secondary metabolites, heat-shock proteins, and pathogenesis-related protein were also elucidated to understand drought-resistance related gene induction. Therefore, the transcriptional activity of ROS along with antioxidant enzymes, stress-related proteins, proline and secondary metabolites were strongly induced and played the tolerance-related role in response to drought stress. Moreover, an increased level of abscisic acid (ABA) and brassinosteroids (BRs) at physiological and transcriptomic level proved the fact that both hormones have a key defensive role against drought stress in grapevine leaves. In general, these findings will provide the drought tolerance-related gene information, which could be further used for developing drought-resistant grapevine varieties.

Keywords: Transcriptome, Drought-stress, Grapevine, Chlorophyll, Secondary metabolites, Proline metabolism

Introduction

Grapevine (*Vitis vinifera* L.) is an economically important crop, having 7.8 million hectares of cultivated land with an annual production of 67.6 million tons worldwide (Griesser et al. 2015). The world climate change pattern has influential effects on the survival and productivity of grapevine. Thus, growth and development of grapevine are consequently affected by abiotic stresses, such as drought, salinity, severe temperature, chemical toxicity and oxidative stress. Among these factors, drought has the deleterious effects on crop productivity, genetic potential and reproduction worldwide (Ma et al. 2012; Krasensky and Jonak 2012). Globally, approximately 45% of the agricultural lands are under constant or periodic water shortages (Ashraf and Foolad 2007), finally resulting in nearly 50% of yield losses. Plant response to these limited water conditions is mediated by expression of numerous genes encoding stress-related proteins and enzymes functioning in the biosynthetic pathways of osmolytes and other stress-related metabolites (Vinocur and Altman 2005). The elucidation of these metabolic pathways is very important to identify stress tolerance genes for further functional analysis. The stress-inducible genes under osmotic stress in plants are categorized into two groups: those protect plants by encoding protein functions, and those encoding regulatory proteins (Shinozaki et al. 2003; Valliyodan and Nguyen 2006). Nevertheless, drought induces free radicals affecting reactive oxygen species (ROS) and reduces the chlorophyll contents. Plants being sessile are capable of making adaptive changes in phenology, physiology and morphology is called phenotypic plasticity that allow it to tolerate drought stress including, slow photosynthesis and cell division, stomatal closure to balance the uptake and loss of cellular water potential, enhancement in cuticle thickness and root growth, but these adaptations are inadequate to restore physiological water potential contained by the cell (Khan 2011).

Water scarcity is a threatening factor for not only to viticulture productivity, but also for berry development and wine quality (Chaves et al. 2007; Escalona et al. 2013). SCHULTZ (2000) proposed that rise in atmospheric CO_2 is leading to rising the environmental temperature, thus leading to scarcity of available soil water for viticulture. Being sessile, grapevine possesses the unique molecular machinery which enables the adjustment by mitigating and adapting the abiotic stress conditions. Therefore, it adjusts the flow of water to leaf and then to the atmosphere by vessel anatomy (Lovisolo et al. 2002), stomatal conductance (Soar et al. 2006),

aquaporin (Vandeleur et al. 2009). Consequently, the slow leaf and shoot growth, elongation of tendrils, inhibition of internodes extension, leaf enlargement, the decline of an average diameter of xylem vessels (Lovisolo et al. 2002) and a minor stimulation in root (Dry et al. 2015) growth under drought is observed in grapevine.

Recently, a novel technique RNA-sequencing (RNA-seq) that implicate deep-sequencing technology to achieve transcriptomic profiling of both model and non-model plants by using different tissue sources responding to different environmental states. This novel approach enables researchers to perceive both identified and novel features in a single assay, allowing the detection of transcript information, allele-specific gene expression, single nucleotide variants and other types, without the availability of prior expressed sequence tags (ESTs) and gene annotations. Moreover, transcriptome data have also been used in characterizing large-scale genes governing the complex interaction and metabolic processes of plant under stress (Silva and Gerós 2009). Though, the drought-regulated stress-response mechanism in grapevine has not been studied in detail so far. This study will provide the basis for selection of drought tolerant genes for grapevine breeding programs (Touchette et al. 2009).

The instant discovery of one step PCR (novel gene detection technique), enables researchers to apply the gene transcriptional data into the practical production because of its simple, sensitive and accurate gene expression technique. However, the perdition of the corresponding phenotype is easier by detecting the expressional changes of genes before advancing to morphological changes. Thus, this technique has much importance in crop production, specifically the countries which are badly impressed by the biotic or abiotic stresses. Hence, different fertilization trials in grapevine at flowering and fruit developmental stages were analyzed and verified by this technique (Wang et al. 2014). Moreover, this technique has its extensive uses in the study of medicine to predict various human diseases (Ibrahim 2011; Lu et al. 2005). The purpose of this experiment was to analyze the drought responded grapevine leaves by transcriptomic analysis, which provides deep insight into the drought stress-responsive mechanisms in grapevine.

Materials and Methods

Plant material and drought treatments

Two-year old ‘Summer Black’ (hybrids of *V. vinifera* and *V. labrusca*) pot grown grapevine plants were selected as experimental material and grown under standard greenhouse condition (25±5°C) at the Nanjing Agricultural University, Nanjing, China. Grapevine plant was subjected to drought (20 days interval) against control, each with two replicates; as it can affect the several physiology and growth related traits in preliminary experiments. Randomly selected mature leaves (two leaves) were collected from both control and drought treated plants (each with three replicate) with the interval of 5 days from 0-20 days and the samples were immediately put in liquid nitrogen and then stored at −80°C until analysis.

Determination of important physiology-related traits

The chlorophyll a and b contents were determined using spectrophotometer at 663 and 645 nm. 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. 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. (2013). Three replicates 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 ± standard deviation (SD). 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. (2015).

RNA extraction, cDNA library construction and Illumina deep sequencing

Total RNA samples of both control and drought-stressed were extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) (A 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. Each sample yielded more than 7.56 GB of data.

Analysis of gene expression level

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 RPKM value computed from SAM files. Gene expression difference 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. We simply defined genes with at least 2-fold change between two samples and FDR (false discovery rate) less than 0.001 as differential expressed genes. Transcripts with $|\log_2 FC| < 1$ were assumed to have no change in expression levels.

Illumina RNA-seq results validation by qRT-PCR

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

Results

To explore the transcriptomic profile of grapevine (*Vitis vinifera* L.) under drought stress, two cDNA libraries were constructed from the mRNA of both control and drought-treated leaf samples, while Illumina deep-sequencing HiSeq™ 2000 was used to perform sequence analysis. The data was submitted to National Center for Biotechnology

Information (NCBI) and can be accessed by Short Read Archive (SRA) database under accession number SAMN04914490. After removing the raw reads and low-quality sequences from both control and drought-stressed leaf samples, finally generated 42.47 and 53.05 million clean reads, respectively, corresponding to 7.56 GB raw data (Supplementary: Table S1). The dataset originated from both control and drought-stressed leaf samples were represented by about 50 million reads, a tag density optimum for quantitative gene expression analysis. Furthermore, the sequence reads were aligned to the grapevine reference genome by using software (soap2/SOAPaligner; http://soap.genomics.org.cn), enabling almost two base mismatches. The total mapped reads (73.44%) matched with both unique (72.01%) and multiple (1.44%) genomic positions (Supplementary: Table S1).

Transcriptomic sequencing as a proficient tool used to equate gene expression levels in different types of samples. In current experiment, the sum of 12451 transcripts was expressed under drought treatment ($|\log_2 Ratio| \geq 1$) and false discovery rate ($FDR \leq 0.001$); whereas, 7983 (64.11%) were up-regulated and 4468 (35.88%) were down-regulated in both control and drought treated grapevine leaf samples (Supplementary: Table S2). Out of 19826 and 22195 expressed genes, the number of 258 and 646 novel transcripts were recorded in both control and drought treated grapevine leaf samples, respectively.

Gene ontology and KEGG analysis of differentially-expressed genes

For the functional characterization of DEGs in control and drought stress treatment, GO (Gene Ontology) based enrichment tests were applied according to the Nr annotations. This GO term includes mainly cellular components, molecular function and biological process. A sum of 12451 (72.11%) transcripts were annotated and classified into 53 functional groups, including 22 in biological process, 17 in cellular component and 15 in molecular functions (Supplementary: Table S3 and Fig. S1**)**. Under the biological process, “metabolic process” with 4537 (75.7% out of 5994; GO: 0008152) transcripts, “cellular process” with 3632 (60.6% out of 5994; GO: 0009987) and “single-organism process” with 3185 (53.1% out of 5994; GO: 0044699) transcripts were predominant. In cellular component, “cell” with 3247 (82.4% out of 3940; GO: 0005623) transcripts, “cell part” with 3247 (82.4% out of 3940; GO: 0044464) transcripts and “organelle” with 2358 (59.8% out of 3940; GO: 0043226) transcripts was observed. Finally,

“catalytic activity” with 4291 (71.3% out of 6021; GO: 0003824) and “binding” (58.1% out of 6021; GO: 0005488) were recorded in molecular functions.

For better understanding the biological functions, KEGG annotation were searched against KEGG database and 7660 (43.99% out of 17412) transcripts were allocated to 306 KEGG pathways (Supplementary: Table S4). Several DEGs under drought were recorded in current study, mainly involved in “metabolic pathway” (2126 transcripts), “biosynthesis of secondary metabolites” (1160 transcripts) and “plant-pathogen interaction” (756 transcripts).

Chlorophyll degradation and photosynthetic competences of grapevine under drought stress environment

Chlorophylls (Chls) are the abundant pigments in biosphere which participates in the initiation of photosynthesis by harvesting light energy and driving electron transport (Fromme et al. 2003). Drought stress had significant effect on the Chl contents and photosynthesis process in leaves. In this experiment, Chla and Chlb contents, photosynthesis rate, stomatal conductance, and CO_2 assimilation rate had marked reduction under drought stress environment. During the 20 days of drought treatment, clear reduction in Chla contents (0.43 ± 0.11 - 0.38 ± 0.06 mg/g) and Chlb contents (0.73 ±0.06 - 0.67 ±0.04 mg/g) photosynthesis (24.67 ± 0.81 - 17.08 ± 0.75 µmole $m^{-2}sec^{-1}$), stomatal conductance (0.15 ± 0.03 - 0.11 ± 0.04 µmole $m^{-2}sec^{-1}$), and CO_2 assimilation rate (9 ± 0.03 - 5 ± 0.03 µmole $m^{-2}sec^{-1}$) was recorded in control and drought-treated grapevine leaves, respectively (Table 1). Similarly Chla synthesis was affected more than Chlb under drought stress, and ultimately affected the photosynthesis activity and gaseous exchange through stomata.

Chlorophyll (Chl) degradation pathway mainly undergoes through three different phases: (I) Chla synthesis from glutamate, (II) the conversion of Chlb into Chla (chlorophyll cycle), and (III) Chl degradation pathway (Hörtensteiner and Kräutler 2011). In the grapevine transcriptome, 27 transcripts involving in chlorophyll metabolic pathway responded differently to drought stress compared with control, of which 17 transcripts were up-regulated and 10 transcripts were down-regulated. However, out of 23 transcripts, 9 transcripts in Chla synthesis (GLTL, Glutamate tRNA Ligase; RAM1, Radical S-adenosyl methionine domain-containing protein 1; PPOX, Protoporphyrinogen oxidase; DHR, Dehydrogenase/reductase SDR family member; POR,

Protochlorophyllide oxidoreductase, and four transcripts of SCD, Short chain dehydrogenase, TIC32) were significantly up-regulated; whereas, 6 transcripts (2 transcripts of HemA, Glutamate tRNA reductase 1; PPOX1, Proporphynogen oxidase 1; CHLH, Magnesium chelatase H subunit; POR, Protochlorophyllide oxidoreductase and SCD, Short chain dehydrogenase) were significantly down-regulated. Meanwhile, Chlorophyllide a oxygenase, (CAO) and Chlorophyll(ide) b reductase NYC1 (CBR) were increased during chlorophyll cycle. Whereas, Chlorophyllase-II, COR2; Pheophorbide a oxygenase, PAO; and Protochlorophyllide-dependent translocon component 52, PDT, were significantly up-regulated and 3 transcripts of Chlorophyllase-I, COR1 were down-regulated during the Chl degradation process (Table 2, Fig, 1, Supplimentary: Table S5). The expression level of VIT_08s0007g08540.t01 (307.93-106.65 RPKM) and VIT_19s0014g03160.t01 (1360.37-307.58 RPKM) revealed high profusion in Chla synthesis pathway (Supplementary: Table S5). Our findings unveiled that drought treatment suppressed the chlorophyll contents because the chlorophyll synthesis was inhibited as degradation pathway was induced by drought stress. Moreover, phytochromobilin synthesis was also affected by the application of drought. The expression of Ferrochelatase-2 (VIT_07s0031g03200.t01, $|\log_2 FC| = 3.032$), Heme oxygenase 1 (VIT_11s0016g05300.t01, $|\log_2 FC| = 2.403$), Heme oxygenase 2 (VIT_18s0001g11040.t01, $|\log_2 FC| = 2.249$) and Phytochromobilin:ferredoxin oxidoreductase (VIT_06s0009g03770.t01, $|\log_2 FC| = 1.705$) was also induced by drought stress (Table 2, Fig, 1, Supplimentary: Table S5).

In grapevine transcriptome, a sum of 23 genes related to photosynthesis pathway, including PSII (5), PSI (2), cytochrome b6-f complex (4), photosynthetic electron transport (4), F-type ATPase (4), photosynthesis-antenna proteins (4) were recorded sensitive to drought stress (Table 2, Supplementary: Table S6). Among the 5 differentially expressed genes in PSII, two psbBs, two psbCs and one psbW were significantly down-regulated compared with the control (Table 2, Supplementary: Table S6). psbC (VIT_00s0396g00010.t01, 280.56 - 1.32 RPKM) possessed the high expression abundance (Supplementary: Table S6). Moreover, expression levels of two psaB in PSI and two transcripts related cytochrome b6-f complex (one petA and one petC) revealed significantly reduction, while two transcripts of petC were found to be increased with control group (Table 2, Supplementary: Table S6). Similarly, the genes involved in the photosynthetic electron transport, two transcripts of petF (VIT_12s0035g00270.t01 and VIT_06s0080g00410.t01) showed down-regulation and two transcripts of petH

(VIT_04s0023g03510.t01 and VIT_10s0003g04880.t01) showed up-regulation compared with the control (Table 2, Supplementary: Table S6). Finally, five genes of F-type ATPase (ATPF1B, ATPF1A, ATPF1G and ATPF0C) and two genes of photosynthesis-antenna proteins (LHCB1, LHCB2, LHCB3 and LHCB6) were significantly down-regulated compared to control under drought (Table 2, Supplementary: Table S6).

Reactive oxygen species (ROS) synthesis and scavenging system under drought stress environment

Overproduction and over-expression of various antioxidant enzymes in the reactive oxygen species (ROS) scavenging system could occur in almost all type of environmental stress conditions (Leng et al. 2015). Drought stress had regulated the effectivity of many antioxidant enzymes activities such as, MDA (5.35 - 8.61 nmol/g), SOD (371.56 - 650.85 U g^{-1} min^{-1}), POD (18.23 – 43.9 U g^{-1} min^{-1}) and CAT (6.32 – 19.01 U g^{-1} min^{-1}) in comparison with the control (Table 1). This enhancement is enzyme activities revealed their role ROS scavenging system. In ROS transcriptomic analysis, many encoding genes were regulated under drought stress; having their involvement in protein synthesis and ROS synthesis and detoxification mechanism. For instance, one NADPH respiratory oxidase and five amine oxidases were up-regulated, proposing their possible role synthesis of ROS under drought stress (Table 3, Fig 2). In ROS scavenging system, 60 differentially expressed genes were identified that were categorized into Fe superoxide dismutase (Fe-SOD, 2 transcripts), peroxidase (POD, 6 transcripts), catalase (CAT, 3 transcripts), glutathione-ascarbate cycle (GSH-AsA, nine transcripts), glutathione peroxidase (GPX, one transcript), glutathione S-transferaze (GST, 26 transcripts), peroxiredoxin/thioredxin pathway (Prx/Trx, eight transcripts), alternative oxidases (AOX, 3 transcripts) and polyphenol oxidase (PPO, 2 transcripts) (Table 3. Supplementary: Table S7).

SODs are classified into three different types depending on metal co-factors: iron (Fe-SOD), copper-zinc (Cu/Zn-SOD) and manganese (Mn-SOD); found in different cellular compartments. They provide defensive mechanism against toxicity of superoxide radicals by rapid disproportionate O_2^- into O_2 and H_2O_2. In our findings, two Fe-SODS were up-regulated, but both genes showed low expression abundance. Perhaps, for Cu/Zn-SOD were significantly down-regulated ($|\log_2 FC| < 1$) (Supplementary: Table S7). Similarly, CAT and POD are the oxide donors during metabolism of H_2O_2. In this study, three CATs and two PODs were up-

regulated, revealing their worth in ROS scavenging system under drought stress (Table 3. Supplementary: Table S7). All three up-regulated CAT genes (VIT_18s0122g01320.t01, from 2888.01 to 358.79 RPKM; VIT_00s0698g00010.t01, from 428.21 to 106.90 RPKM; VIT_04s0044g00020.t01, from 767.21 to 263.73 RPKM) showed high expression abundance; whereas, all up/down-regulated POD genes showed moderate or low expression abundance (Supplementary: Table S7). Additionally, 9 GSH-AsA (5 up-regulated, 4 down-regulated), 27 GPX-pathway (23 up-regulated, 4 down-regulated), eight Prx/Trx (5 up-regulated, 3 down-regulated), three AOX (2 up-regulated, one down-reulated) and two PPO (down-regulated) genes were identified, proposing their key defensive role in against ROS and scavenging H_2O_2 drought stress (Table 3. Supplementary: Table S7).

Plant hormone signal transduction pathway under drought stress environment

Plant hormones play key defensive roles under stress conditions, from which the plant may attempt to escape by enduring various physiological functions. Normally, plant hormones act as central integrators that connect and re-program the composite developmental and stress adoptive signaling cascade (Ruzza et al. 2014). In this study, the hormonal level, including auxin (IAA; 1.373 ± 0.02 - 1.626 ± 0.03 ng/g FW), abscisic acid (ABA; 0.257± 0.01 - 0.908± 0.01 ng/g FW), jasmonic acid (JA; 1.451 ± 0.03 - 1.67 ± 0.05 ng/g FW), gibberellin (GA; 1.53 ± 0.02 - 1.671 ± 0.02 ng/g FW), and brassinostroids (BR; 1.073 ± 0.01 - 1.091 ± 0.01 ng/g FW) was notably increased from control to drought-treated grapevine leaves, respectively (Fig 3).

In grapevine transcriptome, several genes related AUX, GA, ABA, JA, ET (ethylene), and BR signal transduction pathways and synthesis were detected (Supplementary: Table S8). These hormones played pivotal role in grapevine defense against drought stress. Under AUX signaling, three genes (down-regulated) related to auxin transport, eleven auxin response factors (ARF; 7 up-regulated and 4 down-regulated) involved in the transcriptional repressor were detected. Moreover, fifteen genes in auxin induced and responsive proteins (Aux/IAA; two up-regulated and 13 down-regulated), six IAA synthetase (GH3; one up-regulated and 5 down-regulated) and seventeen genes related to auxin and IAA induced proteins (SAUR; 5 up-regulated and 12 down-regulated) were detected in grapevine under drought stress (Supplementary: Table S8). Two natural receptors were up-regulated while four DELLA proteins were down-regulated in the GA under drought. Three ABA responsive proteins (down-regulated), two SNF1-related protein

kinases 2 (SnRK2; up and down-regulated), three PP2C group (up-regulated) genes and six transcription factors (ABF, up-regulated) were involved in abscisic acid pathway under drought (Supplementary: Table S8). Six transcripts of jasmonate-ZIM-domain proteins (one up-regulated and 5 down-regulated) and single jasmonoyl isoleucine conjugate synthase 1 (JAR1; up-regulated) were found in JA hormonal signalling. Moreover, 12-oxophytodienoate reductase 2-like (OPR; up-regulated), linoleate 13S-lipoxygenase 2-1 (LOX; up-regulated) and allene oxide synthase (AOS; down-regulated) were identified in JA pathway under drought stress (Supplementary: Table S8). Three ethylene-responsive transcriptional factors (ETR; 3 up-regulated) being crucial to ET, five ethylene response factor (down-regulated) and three ACC oxidases (CCO; up-regulated) were perceived under drought treatment (Supplementary: Table S8). Finally, two transcripts related BRASSINOSTEROID INSENSITIVE1 (BAK1, up-regulated), ten (down-regulated) brassinosteroid-regulated proteins (BRU1) and 9 (down-regulated) D-type cyclins were identified in brassinosteroid pathway under drought stress (Supplementary: Table S8).

Proline metabolism under drought stress

Proline is known to confer drought stress tolerance by playing diverse roles, *i-e*, stabilizing the membranes and sub-cellular structures (Van Rensburg et al. 1993), proteins and protecting cellular functions by ROS species. Perhaps, it was also reported that proline transport processes play key role in plant adaptation to drought stress. The proline level was increased from 1.624 ± 0.04 - 1.711 ± 0.05 ng/g FW) control to drought-stressed grapevine leaf samples (Table 1). In transcriptomic experiment, sum of 18 genes, including pyroline-5-carboxylate synthetase (P5CS; up-regulated), proline dehydrogenase (PDH; up-regulated), Proline methyltransferase □-Glutamyl kinase (□-GK; up-regulated), Glutamic-□-semialdehye dehydrogenase (GSDH; up-regulated), Pyrroline-5-carboxyate dehydrogenase (P5CDH; up-regulated), Prolyl hydroxylase (4 up-regulated), Acetyl-CoA: glutamate N-acetyl transferase (Ac GACT; 2 up-regulated), N-Acetylglutamate kinase (Ac GK; up-regulated), Acetyl glutamic-□-semialdehyde dehydrogenase (Ac GSD; up-regulated), Acetyl ornithine aminotransferase (Ac OAT; up-regulated), Acetyl ornithine deacetylase (Ac Oam DH; 2 up-regulated), Arginino succinate lyase (ASL; up-regulated) and Arginase (ARG; up-regulated) were identified related to proline metabolism pathway as compared to control (Fig, 4, Supplementary: Table S9). The up-regulation of all the

genes related to proline synthesis and degradation suggested that proline was the key factor in grapevine tolerance.

Biosynthesis of secondary metabolites under drought stress

Plant secondary metabolites are the compounds with no specific role in the maintenance of plant processes, but actually important for the adaptation and defense of plant to cope with its environment. In this transcriptomic study, 73 secondary metabolites related genes linked with shikimate acid (9), alkaloid (2), anthocyanin (33), lignin (21) and terpenoid (8) were recognized under drought treated grapevine leaves (Table 4, Supplementary: Table S10).

Shikimate acid (SA) has its key role in the synthesis of various secondary metabolites such as, alkaloids. In this study, SA pathway possessed one up-regulated 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase 03 (DAHPS3), two down-regulated 3-dehydroquinate dehydratase/shikimate dehydrogenase (DHQ/SDH), one down-regulated shikimate kinase (SK), one up-regulated chorismate synthase 1 (CS1), two down-regulated anthranilate phosphoribosyltransferase (AnPRT) and both up-regulated indole-3-glycerol phosphate synthase (IGPS) and tryptophan synthase beta chain 1 (TS1), respectively (Table 4, Fig, 5, Supplementary: Table S10). All SA genes have moderate transcript abundance (Supplementary: Table S10).

In alkaloid biosynthetic pathway, genes related to strictosidine synthase 3 (STR3) and D-amino-acid transaminase (DAT) were down-regulated (Table 4, Supplementary: Table S10). Strictosidine synthase 3 (STR3) is the central enzyme in alkaloid biosynthesis; catalyze the condensation of tryptamine with secologanin to convert strictosidine.

Anthocyanins are the one class of flavonoids and reported to accumulate under drought stress. Out of 33 genes in anthocyanin biosynthesis, 8 genes related phenylalanine ammonia-lyase (PAL; 4-up-regualted and 4-down-regulated), one trans-cinnamate 4-monooxygenase (C4M; down-regulated), two 4-coumarate--CoA ligase-like 9 (up and down-regulated), 13 stilbene synthase (STS; 6 up-regulated and 7 down-regulated), 3 flavonol synthase/flavanone 3-hydroxylase (F3H; one up-regulated and 2 down-regulated), one 1-aminocyclopropane-1-carboxylate oxidase 5 (A1CO5; down-regulated), two dihydroflavonol-4-reductase (DFR; down-regulated), one anthocyanidin reductase (ANR; up-regulated) and one anthocyanidin 3-O-

glucosyltransferase 2 (UFGT; down-regulated) were observed, suggesting that drought strongly induced the anthocyanin biosynthesis (Table 4, Fig, 5, Supplementary: Table S10).

Lignin is an important phenolic polymer plays various important roles to environmental stresses such as, reinforce the secondary cell wall and confer structural integrity to the plant. In grapevine transcriptome, 21 differentially expressed genes were identified in lignin biosynthesis, which were involved in the drought stress. It includes; 9 up-regulated genes related to shikimate O-hydroxycinnamoyltransferase (SOT), aldehyde 5-hydroxylase (Ald5H), two caffeoyl-CoA O-methyltransferase (COMT), cinnamoyl-CoA reductase 1 (CCR1), cinnamyl alcohol dehydrogenase 1 (CAD1), two peroxidase (POD) and laccase (LAC) (Table 4, Supplementary: Table S10); whereas, 12 down-regulated related to two caffeic acid 3 O-methyltransferase (COMT), cinnamoyl-CoA reductase 1 (CCR1), five peroxidase (POD), three laccase (LAC) (Table 4, Fig 5, Supplementary: Table S10).

Finally, terpenoids have their role in plant defense and their interaction to environment. Eight genes were identified involved in terpenoid biosynthesis (Table Supplementary: Table S), from which hydroxymethylglutaryl-CoA synthase (HMGS), 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXPS), isopentenyl diphosphate isomerase II (IPI2) and terpene synthase (TS) were up-regulated, while hydroxymethylglutaryl-CoA synthase (HMGS), 1-deoxy-D-xylulose-5-phosphate synthase (DXPS) and two squalene epoxidase (SE) were down-regulated in drought-stressed grapevine leaves (Table 4, Fig 5, Supplementary: Table S10).

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

Heat shock proteins (HSPs; HSP100s, HSP90s, HSP70s and HSP60s) and small heat-shock proteins (sHSPs) are the stress responsive proteins that play their part as a molecular chaperone, thus protecting plants from the stress damage. In this pathway, 48 differentially-expressed genes were identified in HSPs, including one HSP101 (down-regulated), three HSP90 (one up-regulated and 2 down-regulated), two HSP70 (up and down-regulated), eighteen sHSPs (12 up-regulated and 6 down-regulated), twenty other HSP genes (17 up-regulated and 3 down-regulated) and heat-stress transcription factors (4 up-regulated and one down-regulated) (Table 5, Supplementary: Table S11). The high molecular weight HSPs (HMW HSPs), including HSP90s

and HSP70s were up-regulated (Table 5, Supplementary: Table S11). One up-regulated transcript of HSP70s was expressed at high abundance (VIT_17s0000g03310.t01; 650.81 to 532.18 RPKM), compared with other HMW HSPs (Supplementary: Table S11). The up-regulated, VIT_16s0098g01060.t01 (from 706.59 to 1.98 RPKM) from sHSPs and VIT_14s0060g01490.t01 (from 363.93 to 355.88 RPKM) from other HSPs, expressed at moderate abundances, but remaining sHSPs, other HSPs and heat-stress transcription factors showed expressed at low abundances (Supplementary: Table S11).

The regulation of pathogenesis-related protein (PR) plays a pivotal role in plant immune system. In this study, 72 transcripts were identified as differentially-expressed genes, including 10 pathogenesis-related protein PR-1 (4 up-regulated, 6 down-regulated), 9 Beta-1,3-glucanase (PR2; 4 up-regulated, 5 down-regulated), 19 chitinase (4 up-regulated, 15 down-regulated), 14 thaumatin-like protein (PR5; 6 up-regulated, 8 down-regulated), 4 Pathogenesis-related protein 10 (2 up-regulated, 2 down-regulated), 10 non-specific lipid-transfer protein (PR14; 5 up-regulated, 5 down-regulated), four Germin-like protein 2 (2 up-regulated, 2 down-regulated) and two pathogenesis-related transcription factors (2 down-regulated) to code disease resistance proteins (Table 5, Supplementary: Table S11). Conversely to HSPs, most of the PR showed down-regulation in grapevine leaves under drought stress. Moreover, 4 up-regulated transcripts, including PR1 (VIT_03s0088g00890.t01, $|\log_2 FC| = 8.75$), chitinase (VIT_05s0094g00320.t01, $|\log_2 FC| = 8.29$), thaumatin-like protein (VIT_02s0025g04290.t01, $|\log_2 FC| = 3.84$) and Pathogenesis-related protein 10 (VIT_05s0077g01600.t01, $|\log_2 FC| = 8.31$) were only expressed in treatment group (Supplementary: Table S11). Additionally, ten dirigent proteins (3 up, 7 down-regulated) and 13 proline related proteins (6 up, 6 down-regulated) were also recovered from this study (Table 5, Supplementary: Table S11). These results proposed that drought stress badly affected the immune system of plant.

qRT-PCR validation of differentially expressed genes from Illumina RNA-Seq

In order to investigate the accuracy and reproducibility, 16 differentially-expressed genes were selected randomly from RNA-Seq results for quantitative real-time (qRT) PCR, these transcripts represent all the major up/down-regulated functions that were identifies in our transcriptome data including, metabolism, hormone signaling, disease resistance and regulatory proteins. The gene function, primer sequence, RPKM, Log_2 values and qRT-PCR results are presented in

Supplementary: Table S12 and Supplementary: Fig. S2. The qRT-PCR findings of 16 (8 up-regulated and 8 down-regulated) selected genes were consistent with the RNA-seq results, revealing the accuracy and reliability of our RNA-seq results.

Discussion

Drought stress affects many physiological processes of plants such as, photosynthesis, hormone signaling, lipid metabolism and protein biosynthesis. Thus, plants have devised various molecular mechanisms to reduce their utilization of available resources and adjust their growth to mitigate the adverse stress conditions (Osakabe et al. 2014). In this study, various pathways related to chlorophyll degradation, photosynthesis, ROS, hormone signal transduction, proline metabolism, biosynthesis of secondary metabolites, HSPs and PRs were elucidated to explore the severity of drought on grapevine and its drought-tolerance mechanism.

Cholorphylls (Chls) are the principal light-absorbing pigments and key components of photosynthesis in plants. In this research, drought stress has a deleterious effect on the inhibition of photosynthesis process and remarkably decreases the Chl contents at physiological and transcriptomic level (Table 2 and Fig. 1). These findings were in good agreement to some reports proposing drought-induced Chl degradation associated with the decrease in photosynthetic activities (Carmo-Silva et al. 2007; Pontier et al. 2007; Strand et al. 2003). The decrease in Chl contents is mainly due to enhanced Chl degradation process as a result of obstructed Chl synthesis. Moreover, transcriptomic data demonstrated that drought stress inhibited the Chl biosynthesis activity and induce the Chl degradation enzymes such as, HemA and CHLH (Fig. 1, Supplementary: Table S5). The worth mentioning is that HemA (Glutamyl-tRNA reductase 1) and CHLH (Magnesium chelatase H subunit) are considered key enzymes in Chla synthesis, but their activities were decreased, revealing the slow process of Chla synthesis (Hörtensteiner and Kräutler 2011). Another experiment related to Chl synthesis and degradation also verifies this statement (data not published). Furthermore in Chl cycle, the oxygenation reactions of chlorophyll(ide) a to chlorophyll(ide) b are catalyzed by chlorophyllide a oxygenase (CAO) (Sakuraba et al. 2009), whose activity was also decreased under drought stress. In contrast, the chlorophyll(ide) b to a conversion is catalyzed by chlorophyll(ide) b reductase NYC1 (CBR) and its activity was enhanced during Chl breakdown (Scheumann et al. 1999), also support our findings (Fig. 1). Furthermore, PAO (pheophorbide a oxygenase) is regarded as an important Chl

catabolic enzyme (Barry 2009; Harpaz-Saad et al. 2007) and participated well in Chl degradation process as its activity was increased under drought stress (Fig.1 Supplementary: Table S5) .

Meantime, the photosynthetic activity, stomatal conductance and CO2 assimilation rate was significantly decreased in grapevine leaves under drought stress as compared to control (Table 1), and similar findings have also been reported by Leng et al. (2015) and Tombesi et al. (2015). Moreover, the photosynthesis-related genes, involved in PSII, PSI, cytochrome b6-f complex, photosynthetic electron transport, F-type ATPase and photosynthesis-antenna proteins were significantly decreased in drought-induced grapevine leaves (Table 2, Supplementary: Table S6). The primary light-driven photosynthesis reactions are carried out in the thylakoid membrane arbitrated by PSII and PSI (Ferreira et al. 2004), but the extent of light-harvesting proteins (CP47, CP43), which binds the chlorophyll a molecules was decreased (Supplementary: Table S6). Perhaps, PsaB is regarded as the heart of PSI that binds P700 special chlorophyll pair in which light-driven charge separation occurs (Nelson and Ben-Shem 2002) was also down-regulated under drought stress. The F-ATP synthase enzyme which is involved in the catalysis the ATP synthesis was also affected by drought stress. Finally, drought stress gradually decreased the activities of PSII electron transport and light-harvesting complex (photosynthesis-antenna proteins) (Supplementary: Table S6). These results are also in agreement to some previously published research on drought-stress effects on photosynthesis process (Pinheiro and Chaves 2011; Osakabe et al. 2014; Zivcak et al. 2013). No doubt, drought stress induces stomatal closure and influence CO_2 absorption, which in turn impact the photosynthesis process in plant. Collectively, our findings on photosynthesis phenomenon at physiological and transcriptomic level suggested that drought stress definitely affected the primary photosynthesis metabolic process, and the decline in photosynthesis process was connected with the chlorophyll degradation.

ROS is the universal response of the plants against any type of environmental stress to prevent oxidative damage. Drought stress has induced the level of antixidative enzymes (Table 1), are known to play important defensive role under drought environment are validated by (Leng et al. 2015). NADPH oxidase significantly play pivotal role in ROS synthesis and accumulation (Apel and Hirt 2004). In this experiment, one NADPH oxidase and five amine oxidases were significantly up-regulated, proposing their contribution in ROS synthesis under drought stress.

Meanwhile, to minimize the risk of oxidative damage, antioxidative (enzymatic and non-enzymatic) defense system is activated in the plants. Significant increase in CAT, SOD and POD activities suggested that ROS scavenging system actively played defensive role in response to drought stress. SOSODs are regarded as first line of defense against ROS and have two isozymes Fe-SOD and Cu/Zn-SOD in plant chloroplast (Apel and Hirt 2004). It is worth mentioning that both Fe-SODs were up-regulated, but Cu/Zn-SODs were down-regulated, these findings are in agreement with the fact that Fe-SOD replaced the Cu/Zn-SODs under Cu deficit conditions (Leng et al. 2015). Other enzymes, including CAT, POD, GSH-AsA cycle, PPO, GST, AO, MDHAR, DHAR and GR also possess the drought-responsive antioxidative defense system in grapevine (Bolwell 1999). Perhaps, non-enzymatic antioxidants such as, glutathione and proline also enhanced the level of ROS in grapevine in response to drought-stress. This study is consistence with ROS scavenging system under drought stress (Chaves et al. 2009; Ioannidi et al. 2009). Generally, ROS related analytical and transcriptomic findings present the broad spectra to understand their role at cellular level in response drought, which may be helpful in production of ROS tolerant grapevine cultivars by using biotechnological approaches.

Drought stress causes dehydration and inhibition of water in plants. Plant hormones, such as abscisic acid (ABA), auxin (AUX), Gibberellin (GA), ethylene (ET), jasmonic acid (JA) and brassinostroid (BR) accumulate under dehydration condition and play important role in response to stress and tolerance of plant (Yamaguchi-Shinozaki and Shinozaki 2006). ABA activates the subclass III protein kinases of SnRK2 family, which further facilitate the regulation of stomatal apertures and induce drought tolerance (Fujii et al. 2011; Yoshida et al. 2002), favor our findings of increased activity of SRK2I protein kinase under drought stress in grapevine leaves (Supplementary: Table S8). The enhanced activities of PP2C genes during the drought stress in grapevine leaves proposed that PP2C has its primary role in stress tolerance, especially in regulating ABA response (Chan 2012). In contrast, IAA induces stomatal openings, hence all the genes related to IAA and AUX/IAA was down-regulated to prevent stomatal opening during drought stress (Acharya and Assmann 2009). But, drought stress induced the activities of auxin response factors (ARF), which are believed to be involved in tolerance of the plant (Li et al. 2004). Stress environment enhances reduction in bioactive GA, induces DELLA proteins accumulates to inhibit the plant growth, suggesting that DELLA is involved in stress tolerance. The decreasing activity of DELLA in our findings proposed that drought stress ceased the plant

growth as compared to control (Achard et al. 2008; Magome et al. 2008). JA amino acid conjugates (JAR1) are enduringly present in the plant leaves and increased under osmotic stress to induce the defensive mechanism, support our findings (Staswick et al. 2002; Wasternack 2007). Most key enzymes such as, OPR and AOS were increased during plant stress environment (Supplementary: Table S8). Ethylene is regarded as stress hormone because its synthesis is induced under different stress environments. Osmotic stress increases the ethylene production in leaves by up-regulating the synthesis of ethylene precursor 1-aminocyclopropane-1-carboxylate oxidase (ACO), which stimulates plant development and functioning by inducing the diffusion possibility of ABA to its active site (Wilkinson and Davies 2002; Wilkinson and Davies 2008). Furthermore, the expressions of the ethylene-related regulatory genes (ETR1 and CTR1) were intensely increased, which are involved in ethylene biosynthesis, suggesting that ethylene play a positive role as a signaling molecule under drought stress (Schachtman and Goodger 2008). Finally, brassinosteroids (BR) are the only plant steroids, which activate the expression of many genes, especially related to the plant stress environments. Brassinosteroid Insensitive 1 (BR1) plays its functional role in plant growth, morphogenesis and response to environment under drought stress; were found to be up-regulated in our experiment (Supplementary: Table S8). Additionally, BR signal transduction, from cell surface perception to activation of specific nuclear genes will be interesting to investigate in the future.

Plants cope with environmental stress by the accumulation of certain compatible osmolytes such as, proline, which is known to confer the drought tolerance in plants under stress conditions (Kishor et al. 2005) and up-regulation of all the genes related to proline metabolism is the clear evidence of grapevine tolerance in our study. Proline synthesis initiated not only from the glutamate, but also from arginine/ornithine (Fig, 2). Proline biosynthesis commenced with the phosphorylation of glutamate, which then converted into gulatamic-□-semialdehyde (GSA) by Pyroline-5-carboxylate synthetase (P5CS; up-regulated). Similarly, orginine is converted into orthinine by orginase (up-regulated) (Supplementary: Table S9) and then into GSA by the ornithine-δ-aminotransferase (not-detected) enzyme. GSA is then converted into pyrroline 5-carboxylate (P5C) by impulsive cyclization. Finally, proline is synthesized from the P5C by P5C reductase (P5CR) enzyme (Kishor et al. 2005; Lea 1990). In proline degradation pathway, proline is re-converted into P5C by Proline dehydogenase (PDH; up-regulated) and then into glutamate by Pyrroline-5-carboxyate dehdrogenase (P5CDH; up-regulated). Thus PDH and

P5CDH are believed to two most important enzymes in proline degradation to glutamate (Kiyosue et al. 1996; Peng et al. 1996). Hence, proline metabolism signaling may control the gene expression during the drought stress.

In higher plants, various secondary metabolites are synthesized from the primary metabolites such as, amino acids, carbohydrates and lipids and their accumulation occur when plant is subjected to environmental stress. They confer protection against biotic and abiotic stresses (Akula and Ravishankar 2011). Drought stress leads to the cellular dehydration that leads to osmotic stress and removal of water from cellular parts (cytoplasm and vacuole). Shikimate pathway is the only known pathway for the biosynthesis of amino acids and chorismate (Try, Tyr and Phe), also not only act as bridge between central and secondary metabolism, but act as precursor for other secondary metabolites (e.g., phenolics, alkaloids and plant hormones) (Maeda and Dudareva 2012). Additionally, Tyr is a precursor of IAA and both Tyr and Try initiate the synthesis of indole alkaloids and isoquinoline alkaloids, which prevent the plant from oxidative stress (Tanaka et al. 1989). Phe is considered the precursor of secondary metabolites family and PAL participates in phenylpropanoid biosynthesis; a key step towards biosynthesis of stilbenes, flavonoids, lignins and various other compounds (Richard et al. 2000). STS (stilbene synthase) catalyzes the initial step of flavonoid biosynthesis pathway, which has the protective function during the drought stress (Winkel-Shirley 2001). Overall, 4 PAL and 6 STS were significantly up-regulated in our findings, proposing the innate link with drought stress. The respectively, up and down-regulation of 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXCR) and 1-deoxy-D-xylulose-5-phosphate synthase (DXPS) deliberately involved and act as rate limiting enzymes in MEP pathway, also found in cu-stressed grapevine leaves (Leng et al. 2015). Dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP) are the universal 5 carbon precursors found in terpenoid synthesis. It has been reported that one isopentenyl-diphosphate isomerase II (IPI2; up-regulated) can catalyze isopentenyl diphosphate to form dimethylallyl diphosphate and one terpene synthase (TPS; up-regulated) (Fig, 3, Table 4, Supplementary: Table S10) (Jonnalagadda et al. 2012; Tholl 2006). The down-regulation of most of the genes related to anthocyanin, lignin and terpenoid biosynthesis have elucidated the negative role of drought stress on accumulation of secondary metabolites in grapevine leaves.

Heat shock proteins (HSPs) are the ubiquitous stress-related proteins that act as chaperone of denatured proteins and translocate the degradation of damaged protein under various types of environmental stresses. HSP members participate in the protein synthesis, folding, aggregation and transportation at early stage from cytoplasm to different intracellular compartments (Li et al. 2012; Nollen and Morimoto 2002). In this experiment, some higher molecular weight HSPs (HSP101, HSP90 and HSP70) were down-regulated, but most of the genes related to small HSPs (sHSPs; 16-30kDa), other HSPs and heat stress transcription factors (HSFs) were up-regulated (Supplementary: Table S11). These findings proposed that HSPs play different stress-responsive role in plant developmental process. The association-related future study of HSPs with drought will be helpful to investigate in the future. Pathogenesis-related (PR) proteins are derived from plant allergens and act as defense-responsive proteins by increase their expression under pathogen attack and variable stress environments. Depending on the functions and properties, PR-proteins are classified into 17 families such as, beta-1,3-glucanases, chitinases, thaumatin-like proteins, peroxidases, small proteins (defensins and thionins) and lipid transfer proteins (LTPs) (Van Loon 1985; Van Loon and Van Strien 1999). Most of the PR-proteins were down-regulated in our study, suggesting that drought stress posed bad effect on PR-proteins defense response. Contrarily, most of the genes related to dirigent-proteins (DIR), play role in lignin formation (important against drought stress) and proline-related proteins were up-regulated, suggesting their possible defensive-role in grapevine in response to drought stress.

Conclusion

This study has not only identified many differentially-expressed genes between control and drought-stressed, but also provide the drought stress acclimation in grapevine. Comparative transcriptome analysis indicated that drought stress affected the chlorophyll synthesis and photosynthesis by down-regulating many genes and up-regulated many genes related to antioxidant enzymes, proline, secondary metabolites and stress-related proteins. Many genes involved in hormone signal transduction pathways of AUX, ABA, GA, ET, JA and BR were identified in response to drought stress, from which ABA is predominantly involved in the stomatal closure. Generally, the transcripts with up/down-regulated expressions were also recovered from this experiment, which elucidates the complexity of the regulation mechanism in

response to stress. The current challenge to cope is to develop improved drought tolerance grapevine cultivars and this study will set a foundation for clear understanding the complex molecular mechanism involved in cellular responses to drought stress for plant breeders.

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

Physiological and biochemical parameters	**Control**	**Drought treatment**
Chlorophyll contents (mg/g)	1.14 ± 0.08	1.08 ± 0.09
Chla contents (mg/g)	0.43 ± 0.11	0.38 ± 0.06
Chlb contents (mg/g)	0.73 ± 0.06	0.67 ± 0.04
Photosynthesis activity (µmole $m^{-2}sec^{-1}$)	24.67 ± 0.81	17.08 ± 0.75
Stomatal conductance (µmole $m^{-2}sec^{-1}$)	0.15 ± 0.03	0.11 ± 0.04
Net CO2 assimilation (µmole $m^{-2}sec^{-1}$)	9 ± 0.03	5 ± 0.03
MDA contents (nmol/g)	5.35 ±0.21	8.61 ±0.25
SOD activity (U g^{-1} min^{-1})	371.56 ±10.21	650.85 ±15.7
POD activity (U g^{-1} min^{-1})	18.23 ±0.97	43.9 ±1.01
CAT activity (U g^{-1} min^{-1})	6.32 ±1.21	19.01 ±0.99
Proline (ng/g FW)	1.624 ± 0.04	1.711 ± 0.05

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	

Table 2. List of differentially-expressed genes related to chlorophyll degradation and photosynthesis in grapevine perceived during drought stress. psbB, Photosystem II CP47 chlorophyll apoprotein gene; psbC, Photosystem II CP43 chlorophyll apoprotein gene; psbW, Photosystem II reaction center W protein; psaB, photosystem I P700 apoprotein A2 gene; petA, cytochrome f; petC, cytochrome b6-f complex iron-sulfur subunit 1; petF, ferredoxin-3; petH, ferredoxin--NADP reductase, leaf-type isozyme; ATPF1B, ATP synthase CF1 beta; ATPF1A, ATP synthase CF1 alpha; ATPF1G, ATP synthase gamma; LHCB1, chlorophyll a-b binding protein of LHCII; LHCB2, light harvesting chlorophyll A/B binding protein; LHCB3, light-harvesting chlorophyll binding protein 3 gene; LHCB6, chlorophyll a-b binding protein CP24 10A.

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

Table 3. List of differentially-expressed genes related to chlorophyll degradation and photosynthesis in grapevine perceived during drought stress. Rboh, respiratory burst oxidase; AO, amine oxidase; Fe-SOD, Fe superoxide dismutase; POD, peroxidase; CAT, catalase, APX, ascorbate peroxidase; MDAR, monodehydroascorbate reductase; DHAR, dehydroascorbate reductase; GR, glutathione reductase; Grx, glutaredoxin; GPX, glutathione peroxidase; GST, glutathione S transferase; Prx, peroxiredoxin; Trx, thioredoxin; AOX, alternative oxidase, PPO, polyphenol oxidase.

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

Table 4. Elucidation on 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; HMGS, hydroxymethylglutaryl-CoA synthase; DXPS, 1-deoxy-D-xylulose-5-phosphate synthase; IPI2, isopentenyl diphosphate isomerase II; TSE, terpene synthase; SED, squalene epoxidase.

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

Trait name	Description	No. of up-regulated	No. of down-regulated	sum
Heat shock proteins	HSP101	0	1	1
	HSP90	1	2	3
	HSP70	1	1	2
	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

Fig1. 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.

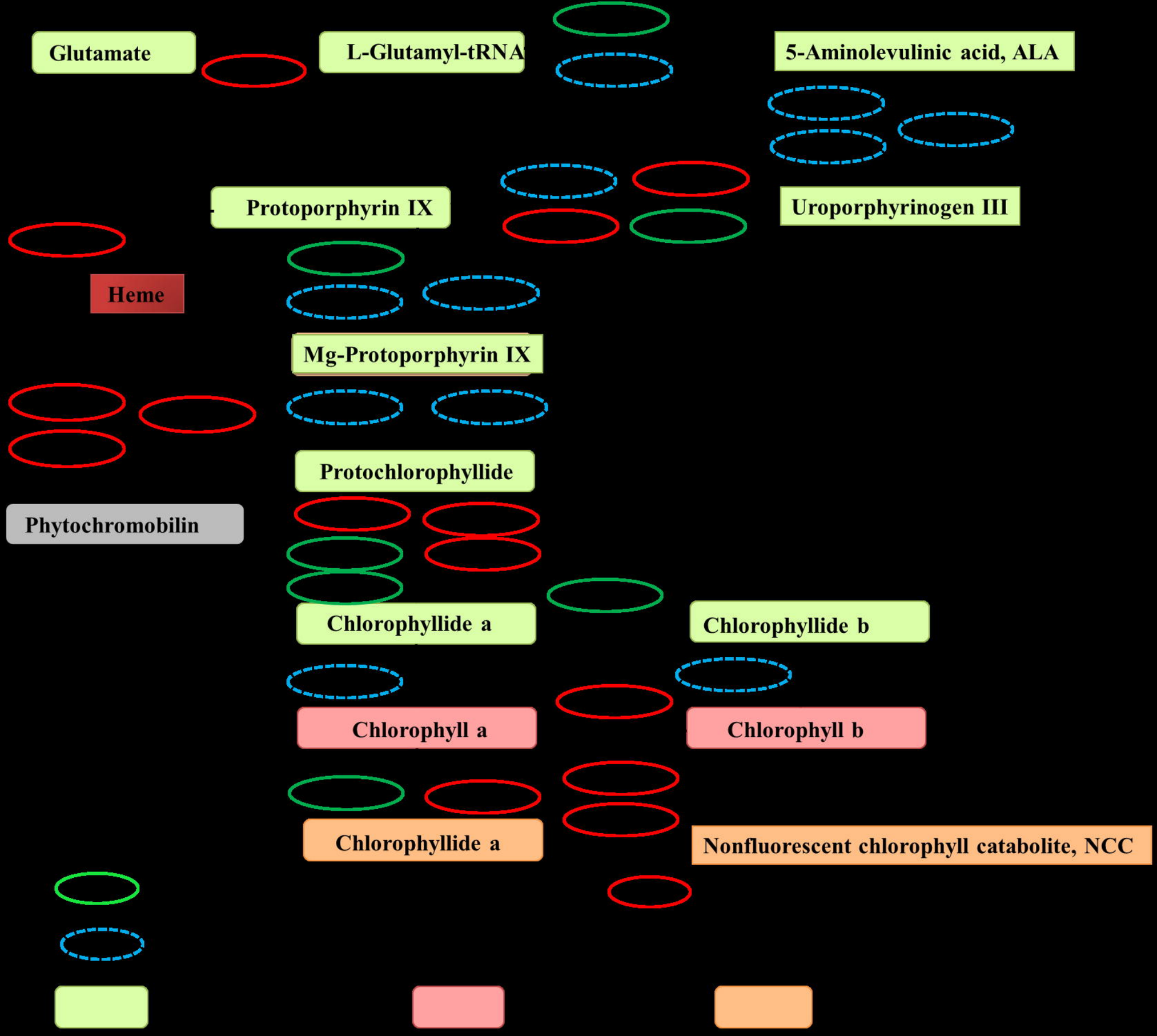
Fig2. Reactive oxygen species (ROS) scavenging pathway in plants. (a) The ascorbate-glutathione (AsA-GSH) cycle, (b) The glutathione peroxidase (GPX) cycle. SOD (superoxide dismutase) initiate the line of defense by converting O_2^- into H_2O_2, ehich is further detoxified by CAT (catalses), APX (ascorbate peroxidases (APX) and GPX (glutathione ascorbate).

Abbreviations: DHA, dehydroascorbate; GSH, glutathione; GSSG, oxidized glutathione; GR, gkutathione reductase; MDAR, monodehydroascorbate reductase; DHAR, dehydroascorbate reductase.

Fig3. 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.

Fig4. 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.

Fig5. 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.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Uroporphyrinogen III
Heme
Mg-Protoporphyrin IX
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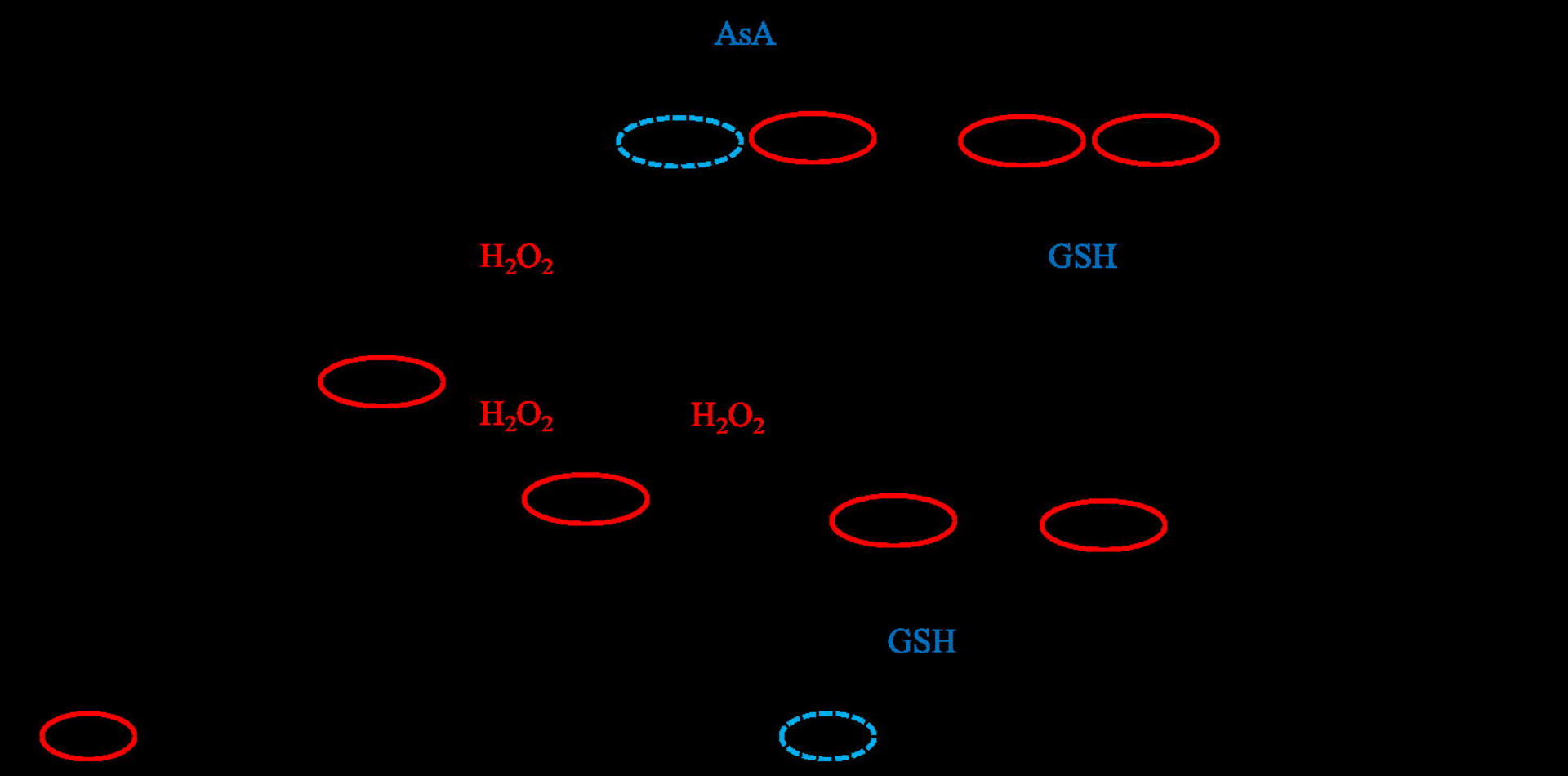
Phytochromobilin
Chlorophyllide a
Chlorophyllide b
Chlorophyll a
Chlorophyll b
Chlorophyllide a
Nonfluorescent chlorophyll catabolite, NCC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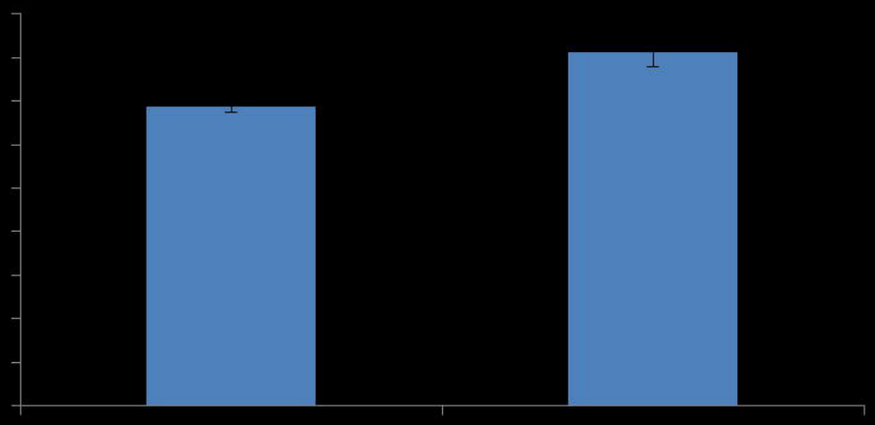

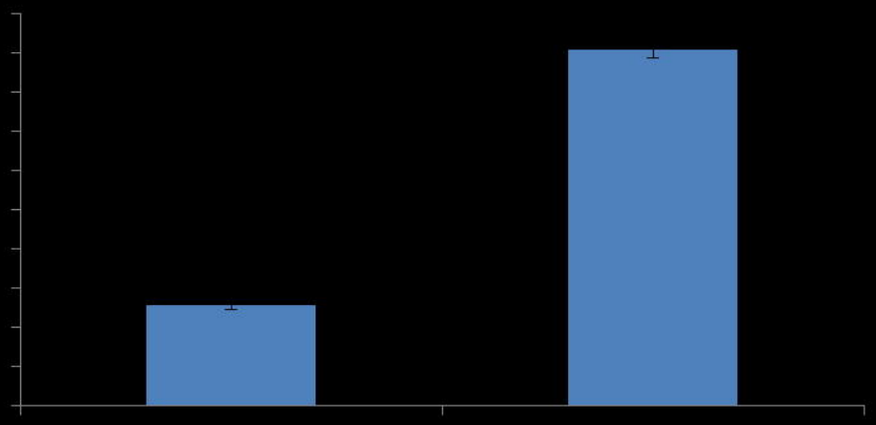

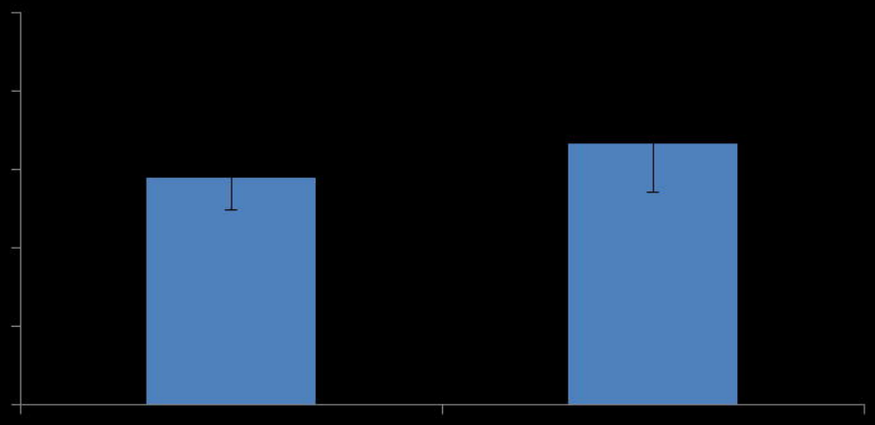

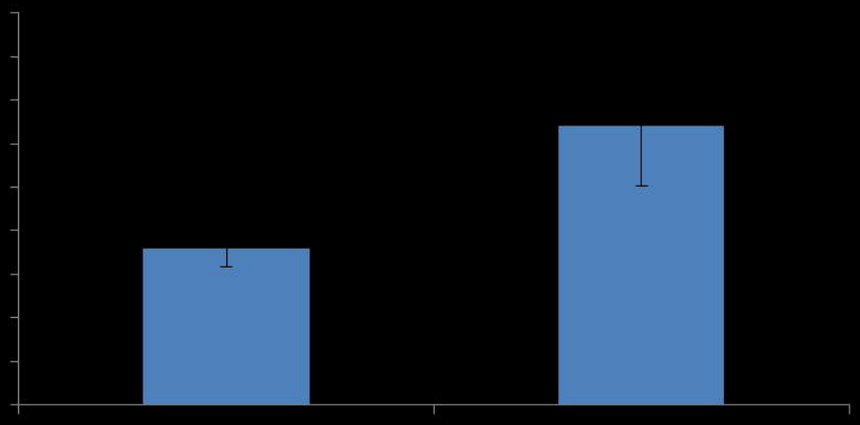

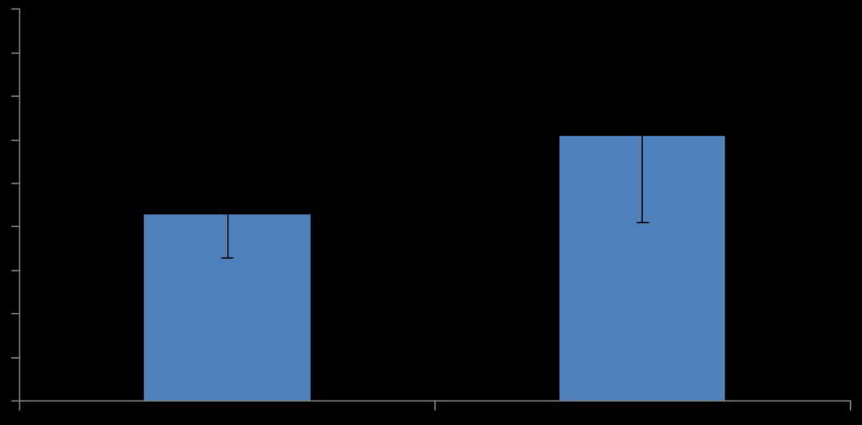

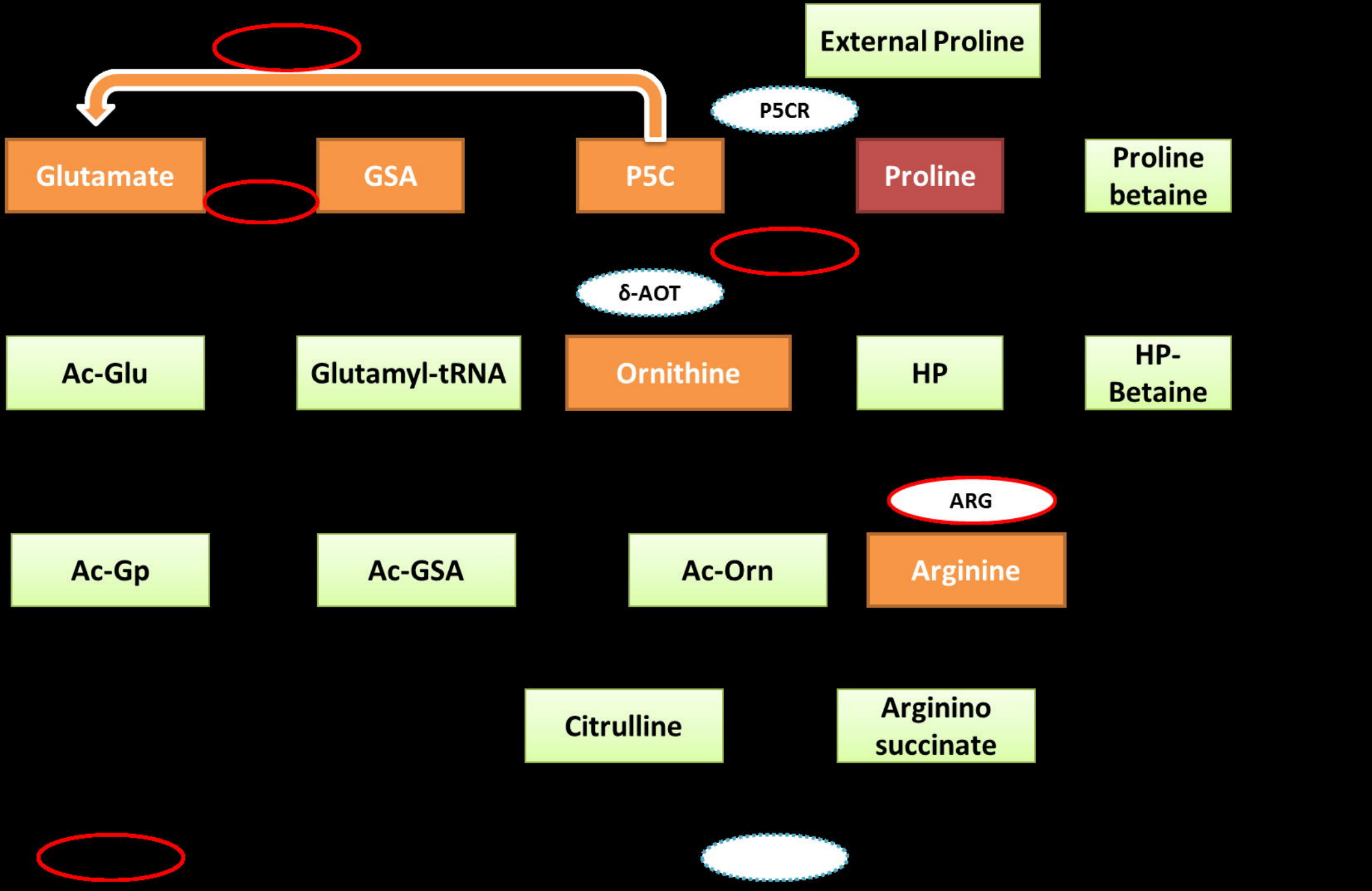
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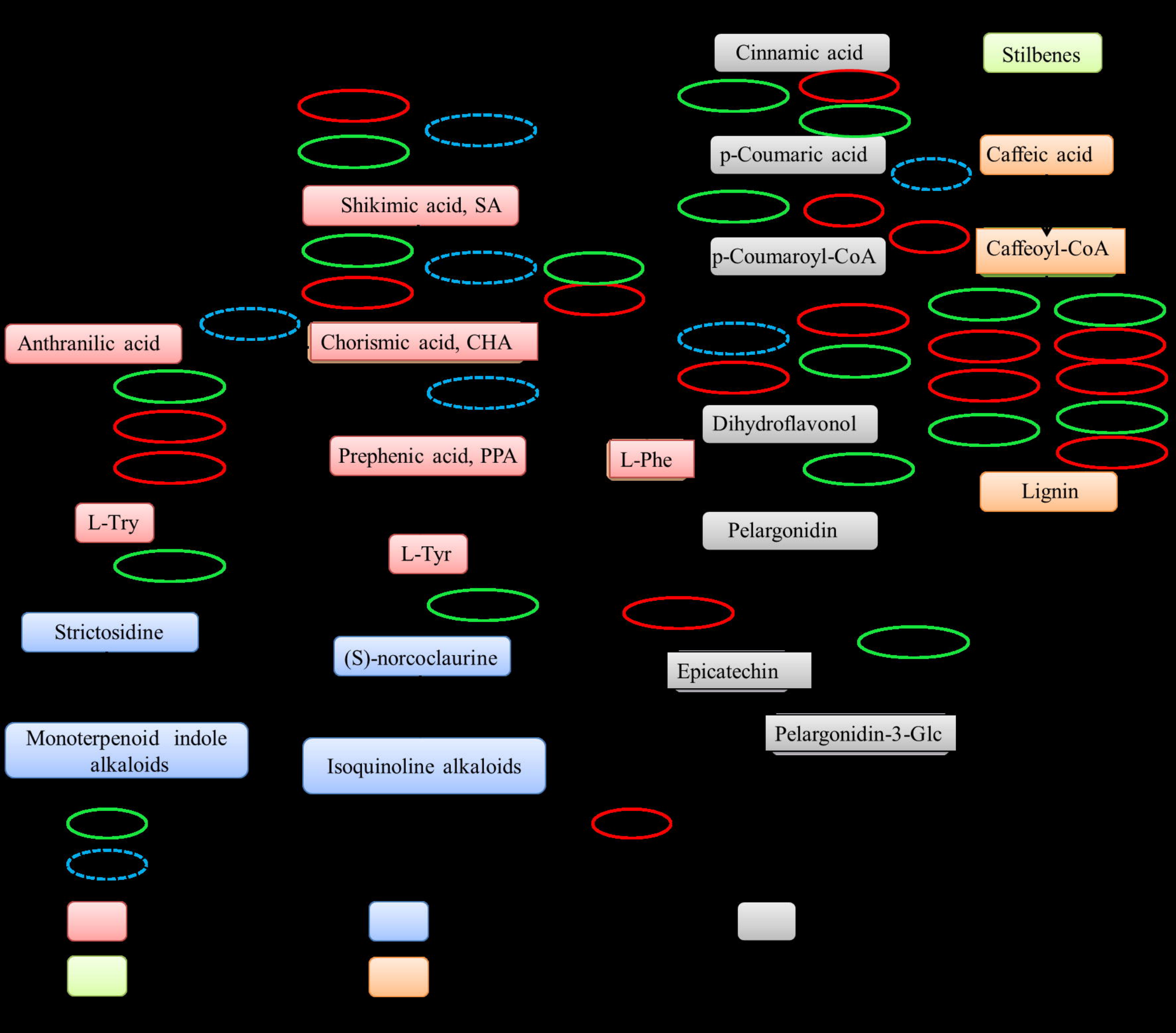
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Prephenic acid, PPA
L-Phe
Lignin
L-Try
Pelargonidin
L-Tyr
Strictosidine
(S)-norcoclaurine
Epicatechin
Pelargonidin-3-Glc
Monoterpenoid indole alkaloids
Isoquinoline alkaloids