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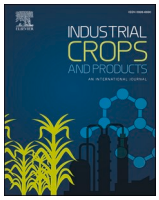

Comparative analysis of drought-responsive biochemical and transcriptomic mechanisms in two *Dendrobium officinale* genotypes

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ABSTRACT

Drought stress is one of the most sever natural disaster, threatening to plant growth and global crop security. *Dendrobium officinale* is a drought-tolerance crop with effective defense mechanisms against drought stress, as well as an important economic plant for medicinal, cosmetic, or ornamental purposes. Revealing the regulatory mechanisms conferring drought resistance upon *D. officinale* is thus crucial for genetic breeding and water-saving agriculture. In this study, the biochemical and transcriptomic profiles of two *D. officinale* genotypes were comprehensively analyzed under three drought stress conditions. The M genotype has a relative weaker drought tolerance, as shown by withered leaves, rapidly accumulated malondialdehyde, and severely repressed expression of photosynthesis-related genes under water deficit. In the O genotype (drought-tolerant genotype), proline content, ascorbate peroxidase and catalase activities significantly increased with intensifying drought stress, showing a higher level than that in the M genotype, especially under severe drought stress. By contrast, superoxide dismutase and peroxidase activities were higher in the M genotype under moderate and severe water deficient. Transcriptome analysis demonstrated that mild water deficit initiated the plant hormone signal transduction pathway, while severe drought stress launched the flavonoid biosynthesis pathway in both *D. officinale* genotypes. Notably, high expression of most protein phosphatases type 2 C in the M genotype, a negative regulator of abscisic acid signaling, may partially explain the relative weaker drought tolerance of the M genotype. Moreover, the higher flavonoid content corresponding with the highly expressed *PAL* and *DFR* in the O genotype than in the M genotype, may confer a stronger drought tolerance upon the O genotype under water deficit. Additionally, the biased expression pattern of heat shock proteins, late embryogenesis abundant proteins, and dehydrins may also be linked to the different drought responses of the two *D. officinale* genotypes. Our results provide a theoretical basis for drought-tolerant crops breeding and water-sparing agriculture.

1. Introduction

Owing to global warming, the frequency, scope, and duration of droughts are increasing, with droughts becoming one of the most serious natural disasters and environmental issues globally. Water deficit inhibits plant growth and development, resulting in stomata closure and a reduction in CO_2 assimilation (Reddy et al., 2004; Khanna-Chopra and Selote, 2007), chlorophyll destruction and chloroplast dismantling (Hassan et al., 2020), suppressed photosynthesis (Sun et al., 2013), lipid peroxidation and DNA changes in plant cells (Noctor et al., 2014), and even death. Moreover, drought stress could result in extensive agricultural productivity losses (Xu et al., 2019). Therefore, it is of considerable importance for the sustainable development of water-saving agriculture to elucidate the mechanisms of drought stress responses and breed drought-tolerant cultivars.

Plants coping with drought stress activate diverse defense systems to

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alleviate stress damage and withstand adverse environmental conditions. For instance, increased antioxidant enzymes, such as superoxide dismutase (SOD), peroxidase (POD), ascorbate peroxidase (APX) and catalase (CAT), and accumulation of non-enzymatic compatible solute like proline and glutathione, prevent reactive oxygen species (ROS) accumulation (Mittler, 2002; Asensi-Fabado and Munné-Bosch, 2010). The critical roles of phytohormones in regulating plant adaptation to drought stress has been demonstrated in many plants. Abscisic acid (ABA) signaling pathway plays a central role in drought responses in plants (Kim et al., 2010). The overexpression of tomato *9-cis-epoxycarotenoid dioxygenase* (*NCED*), a rate-limiting enzyme for ABA biosynthesis, in petunia plants can elevate leaf ABA concentration and induce a considerable increase in drought resistance (Estrada-Meol et al., 2015). Overexpression of *GmERF3*, an ethylene response factor, increases soluble sugar and proline content, and reduces the accumulation of malondialdehyde (MDA) to improve drought tolerance in tobacco plants (Zhai et al., 2017). The positive role of brassinosteroids (BRs) in enhancing drought resistance were found in *Brassica napus*, *Arabidopsis*, and wheat (Kagale et al., 2007). Several stress-associated proteins and peptides were also important regulators in the response to drought stress in plants. The small heat shock protein (sHSP) positively regulates drought, heat, and salt stress tolerance in pepper (Feng et al., 2019). Late embryogenesis abundant (LEA) proteins have been found associated with cellular tolerance to dehydration (Olvera-Carrillo et al., 2010). The CLAVATA3/EMBRYO-SURROUNDING REGION-RELATED 25 (CLE25) peptide modulates stomatal control via ABA in long-distance signaling to prevent water loss (Takahashi et al., 2018). In addition, plants can maintain osmotic pressure in adverse environments through the osmotic regulation of substances such as soluble sugars and free amino acids (Blum, 2017). Collectively, these defense mechanisms often coordinately regulate plant drought tolerance.

Dendrobium officinale (or *Dendrobium catenatum*) belongs to *Dendrobium* in Orchidaceae family with high value in traditional Chinese medicine. Its stems and leaves contain numerous active ingredients, including polysaccharides, flavonoids, and alkaloids, etc. These metabolites exert anti-inflammatory, anti-oxidative, anti-tumor, and anti-aging effects, can improve the immune function (Ng et al., 2012), and have been used as an additive in skin care as antioxidant, skin whitening, and anti-aging agents (Chen et al., 2022; Zhang et al., 2022a). Zhang et al. (2022a) showed that fermented *D. officinale* polysaccharides can protect human skin fibroblasts against UVA-induced photoaging. These active substances are considered valuable, reflected in their use in medicines and industries, and are possibly the result of the adaptation of *D. officinale* to the external environment. *D. officinale* is an epiphytic orchid and grows on trunks and cliffs (Atwood, 1986). Owing to this water-scarce environment, *D. officinale* has evolved sophisticated defense mechanisms to against severe drought stress, such as abundant metabolites and facultative crassulaceaen acid metabolism (CAM) that an evolutionary adaptation of photosynthesis to reduce water loss under drought. Huang et al. (2021) reported that plant hormone biosynthesis and signal transduction, particularly ABA, may play a vital role in regulation of facultative CAM in *D. officinale*. Although attempted by numerous studies, the elucidation of regulatory mechanism conferring drought resistance upon *D. officinale* remain elusive.

Comparison of different responses between different drought resistance genotypes is an effective method for identification the key pathways and genes involved in drought tolerance, which remain poorly characterized in *D. officinale*. In the present study, two *D. officinale* genotypes with different drought tolerance were subjected to three drought stress conditions, and the drought-responsive biochemical profile and gene expression pattern of the two genotypes were compared. This study aims to uncover the regulatory mechanism underlying drought tolerance in *D. officinale*, which has great scientific significance and practical value for molecular breeding of *Dendrobium* plants and water-saving agriculture.

2. Materials and methods

2.1. Plant materials and drought treatment

Two *D. officinale* genotypes of 3-year old plantlets used in this study, designed as M and O, were grown in a mixture of moss and fermented pine bark (1:2). The stem internode length of the two genotype were compared by determination of the upper first to tenth stem internode length of 15 branch per genotype. These plantlets were placed in the greenhouse at a day/night temperature of 28/24 ℃ with a 12-h period, an irradiance of 130–180 μmol m^{-2} s^{-1}, and relative humidity of 75–80%. A total of 80 plantlets per genotype were divided into four groups. Then, water was withdrawn for 0 (control), 7 (mild drought), 20 (moderate drought), and 45 days (severe drought). Samples of the M genotype were designed as M0, M7, M20 and M45, and for the O genotype were designed as O0, O7, O20, and O45. After drought treatment, the upper second to sixth stem internodes from five plantlets were collected and pooled per biological replicate at 9:00–10:00 am, immediately frozen in liquid nitrogen, and stored at − 80 ℃ for transcriptome sequencing and biochemical analysis. A completely randomized block design with three replicates was employed in this study. The water content of stem was determined by drying at 80 ℃ for 48 h in an oven.

2.2. Determination of MDA content

Fresh stem tissues were ground and homogenized in pre-chilled 50 mM potassium phosphate buffer solution (pH 7.8) in a pre-chilled mortar on an ice bath. The supernatant was carefully collected by using pipette after centrifugation at 12,000 × *g* for 15 min at 4 ℃. The supernatant was used to determine the MDA content according to the method suggested by Zhou and Leul (1998) by estimating the amount of thiobarbituric acid-reactive substance (TBARS) which generates from the reaction between MDA and thiobarbituric acid in absorbance at 532 nm.

2.3. Determination of antioxidant enzyme activity and proline content

The supernatant obtained from the above extraction method was directly used for SOD, CAT, POD and APX activities analysis, and proline content determination. These enzymes activities, proline and protein contents were estimated using specific detection kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to manufacturer's instructions on ELISA assay system (Infinite M200 pro, Tecan, Switzerland). SOD activity was determined by measuring the photochemical inhibition of nitroblue tetrazolium (NBT) in absorbance at 560 nm. CAT and POD activities assay were performed based on the degradation concentration of H_2O_2 in absorbance at 240 nm and 420 nm, respectively. APX activity was determined by measuring AsA oxidation rate in absorbance at 290 nm. The concentration of proline was determined in absorbance at 520 nm. The Bradford assay was used for protein content determination in absorbance at 595 nm.

2.4. Determination of total flavonoid content

Fresh stem tissues were ground and homogenized by using 80% methanol to determine the total flavonoid content (TFC). The homogenate was ultrasonic extraction in 25 ℃ water-bath at 43 Hz frequency for 20 min, and shaken for 24 h followed by centrifugation at 12,000 × *g* for 10 min according to the method described in Menichini et al. (2009). The TFC was detected according to the method described in Marinova et al. (2005) with a slight modification. Briefly, 200 μl supernatant or standard solution of quercetin was mixed to 20 μl 5% $NaNO_2$ in a sterile microplate, followed by flicking the tube wall to mix well. After incubation for 5 min, 100 μl 2% (w/v) $AlCl_3$ was added, left to stand for 6 min before adding 100 μl NaOH (1 M). The mixture was gently and thoroughly mixed, and then incubated for 25 min at room temperature.

The TFC was determined on ELISA assay system (Infinite M200 pro, Tecan, Switzerland) in triplicate in absorbance at 510 nm.

2.5. RNA-Seq library construction and sequencing

Fresh stem tissues were ground to a fine powder in liquid nitrogen. And total RNA of fresh stem tissues were isolated using RNAprep Pure kit (DP441, Tiangen, Beijing, China). NanoDrop 2000 spectrophotometer (Thermo Scientific, NY, USA), 1.2% Agarose gels, and Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA) were used to assess the RNA quality and quantity. Qualified RNA samples were used to constructed sequencing libraries following manufacturer's recommendations. And then high-throughput sequencing of these cDNA libraries were carried out on Illumina HiSeq X Ten platform (Illumina, CA, USA).

2.6. RNA-Seq data analysis

Low-quality reads and adapter sequences were removed from raw reads generated by high-throughput sequencing using Trimmomatic (Bolger et al., 2014), followed by sequence quality evaluation using FastQC. High-quality clean reads were mapped to the *Dendrobium officinale* L. reference genome (accession code JSDN00000000) using Hisat2 (Zhang et al., 2016; Kim et al., 2019). And then, the mapped reads were assembled by StringTie (Pertea et al., 2015). The expression abundance of each gene was quantified and normalized into fragments per kilobase of transcript per million mapped reads (FPKM) using RSEM package (Li and Dewey, 2011). DESeq2 software was used to identify differentially expressed genes (DEGs) with absolute $\log_2$ (fold-change) ≥ 1 and false discovery rate (FDR) < 0.05. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed on the annotated DEGs using topGo and clusterprofiler packages, respectively (Kanehisa et al., 2008). The raw data is available in the NCBI databases with the BioProject accession ID PRJNA952313.

2.7. Validation by quantitative reverse transcription-PCR

Total RNAs were extracted as descried in Section 2.5, and used as template to synthesize first-strand cDNA using FastKing RT kit (Tiangen). The primers of those selected genes used in this study were listed in Table S1. The *DcEF-1-alpha* gene was used as the internal reference genes. qRT-PCR was performed using SYBR Green PCR kit (Tiangen) on the Bio-Rad CFX96 Touch q-PCR System (Bio-rad, CA, USA). The $2^{-\Delta\Delta CT}$ method was used to analyze relative transcript abundances.

2.8. Statistical analysis

Data are presented as the means ± standard deviation (n = 3). A Student's *t*-test test was performed using SPSS software to determine the levels of significance ($p < 0.05$). Different letters indicate significant differences at $p < 0.05$ among different drought conditions. Asterisks represent statistical differences between genotypes (* $p < 0.05$, ** $p < 0.01$).

3. Results

3.1. Phenotypic and biochemical changes in D. officinale with drought

There were clear phenotypic differences between the M (left) and O (right) genotypes (Fig. 1**a**). The leaves of the O genotype were smaller and thicker than those of the M genotype. The stem internode length of the M genotype (average length of 1.31 ± 0.09 cm) was longer than that of the O genotype (average length of 1.03 ± 0.07 cm) (Fig. 1c and d). Both *D. officinale* seedlings appeared healthy with green/dark green leaves under control condition. After severe drought treatment for 45 days, in the M genotype, the mature leaves turned yellow and immature leaves appeared curved and wilted. In contrast, leaves of the O genotype remained green (Fig. 1b). The water content of the stem of the M genotype was markedly higher than that of the O genotype under control

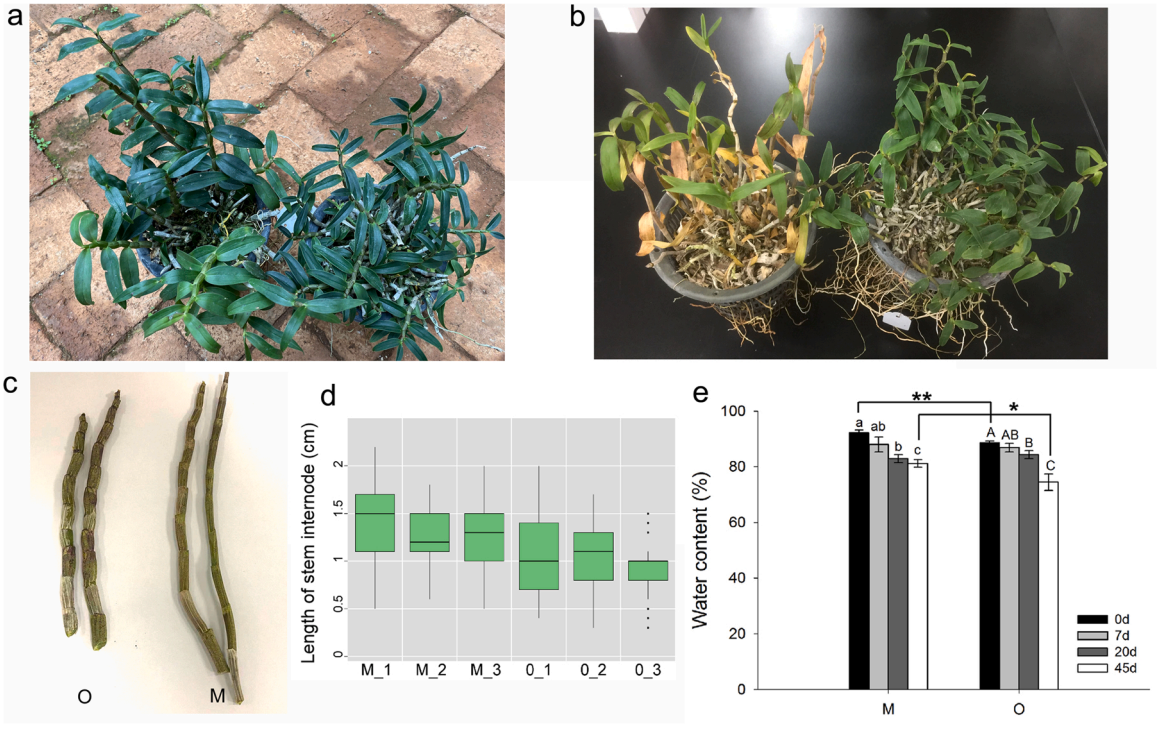

Fig. 1. Comparison of morphology and water contents in the two *D. officinale* genotypes under different drought conditions. a and b, morphology of the two *D. officinale* genotypes under control condition and drought treatment for 45 days. c and d, stem morphology and length of stem intermode under control condition. e, water content of stem. Values with bar are the means ± SD (n = 3). Different letters indicate significantly different at $p < 0.05$ among different drought levels. Asterisks represent statistical differences between genotypes (* $p < 0.05$, ** $p < 0.01$).

conditions. After drought treatment for 45 days, the stem water content of both genotypes declined by 12.47% (in M45) and 15.15% (in O45), respectively compared with the control condition (Fig. 1e). At this point, the stem water content of the M genotype was higher than that of the O genotype.

With intensifying drought stress, the MDA content that is often used as a drought indicator to evaluate the degree of plasma membrane damage markedly increased by 36.38% in M7, 76.09% in M20 and 74.67% in M45 in the M genotype stems. While the MDA content in the O genotype stems remained stable under mild and moderate drought stresses, followed by an increase of 16.98% in O45 compared with control plants (Fig. 2**a**). Under well-watered conditions, there was a considerably higher MDA content in the O genotype than in the M genotype. After drought treatments, the MDA contents did not differ significantly in stems of the two genotypes, which was a result of the markedly increased MDA level in the M genotype. The proline contents in stems of the two genotypes were considerably influenced by drought stress and genotype. With intensifying drought stress, proline contents significantly increased in the two genotypes, except for under mild drought (Fig. 2**b**). Compared with control condition, the proline contents showed a 1.34- and 1.62-fold increase under severe drought stress in the M and O genotype, respectively. The proline contents in the O genotype were significant higher than that in the M genotype, especially under control condition and drought for 20 and 45 days. For example, the proline contents in O45 and M45 were 25.03 ± 0.13 μg g^{-1} FW and 35.90 ± 0.75 μg g^{-1} FW, respectively.

With the extension of drought time, the SOD levels substantially increased and followed by a decrease in the two genotype, showing the highest levels in M20 (65.17 ± 0.22 units mg^{-1} protein) and O20 (52.40 ± 0.12 units mg^{-1} protein) (Fig. 2**c**). Between the two genotypes, the SOD activities in M0 and M7 were considerably lower than in O0 and O7, which was contrast to the SOD activities under moderate and severe drought. For CAT, drought stresses markedly increased CAT activities in the stems of both genotypes. Under severe drought condition, CAT activities increased by 193% and 175% in the M and O genotype compared with control condition, respectively. The accumulation of CAT in the O genotype under mild and severe drought were significant higher than that in the M genotype (Fig. 1d). The change pattern of POD activity was similar with that of SOD activity in the both genotypes throughout the treatment. The POD activities increased and followed by a decrease in the M genotype (Fig. 1e). They retained stable under mild and moderate drought, and then decreased under severe drought in the O genotype. Under moderate drought, POD activity in the M genotype (196.19 ± 5.68 units mg^{-1} protein) was significant higher than that of the O genotype (140.59 ± 1.99 units mg^{-1} protein). For APX, the APX activities sharply increased in the O genotype under three drought stresses from undetectable level to 1.29 ± 0.10 units mg^{-1} protein. However, the APX activities increased under mild drought and decreased with the extension of drought time in the M genotype. Between the two genotypes, the APX activities in M0 and M7 were higher than in O0 and O7. Moderate and severe drought substantially provoked the APX acitivies in the O genotype, and resulted in a significant higher content than that in the M genotype.

3.2. *Drought triggers transcriptome reprogramming in both genotypes*

Transcriptomic changes in the stems under three drought stress conditions were investigated to determine different molecular responses of the two *D. officinale* genotypes. A total of 158.12 Gb clean data were obtained by RNA-Seq sequencing of 24 samples of the two *D. officinale* genotypes (Table S2). Principal component analysis revealed a clear separation between the two genotypes, and a more evident transcriptomic reprogramming in M than in O under different drought stress, particularly in M45 (Fig. 3**a**). Setting fold change ≥ 2 and FDR < 0.05 as thresholds for DEGs, a total of 26556 DEGs were identified. And there were 1854, 2147, 1177, and 2570 DEGs between the two genotypes under drought for 0, 7, 20, and 45 days, respectively (Fig. 3**b**).

Among these DEGs, 58 genes involved in photosynthesis were identified and their expression patterns were shown in Fig. 3**c**. Numerous photosynthesis-related genes were highly expressed under control condition in both genotypes. With intensifying drought stress, the expression levels of many photosynthesis-related genes decreased,

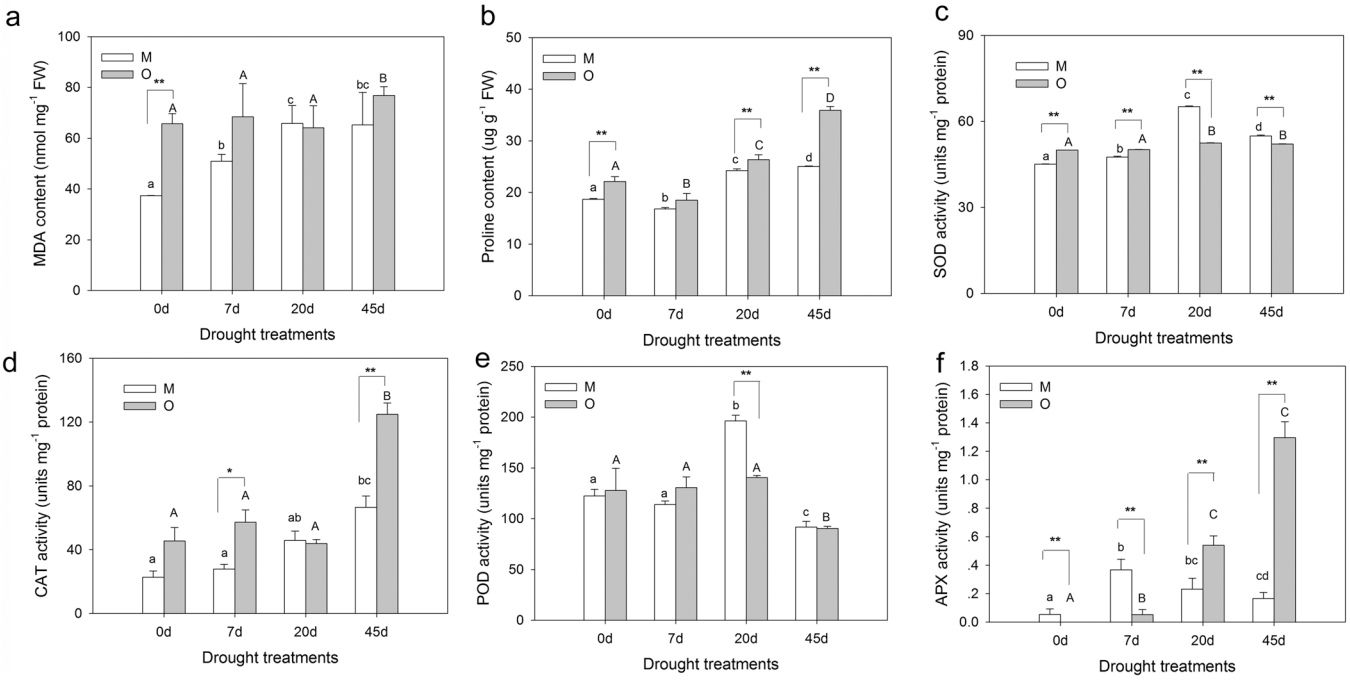

Fig. 2. Changes in malondialdehyde (MDA) and proline contents, and antioxidant enzyme (SOD, CAT, POD and APX) activities in the two *D. officinale* genotypes under different drought conditions. Values with bar are the means ± SD (n = 3). Different letters indicate significantly different at $p < 0.05$ among different drought levels. Asterisks represent statistical differences between genotypes (* $p < 0.05$, ** $p < 0.01$).

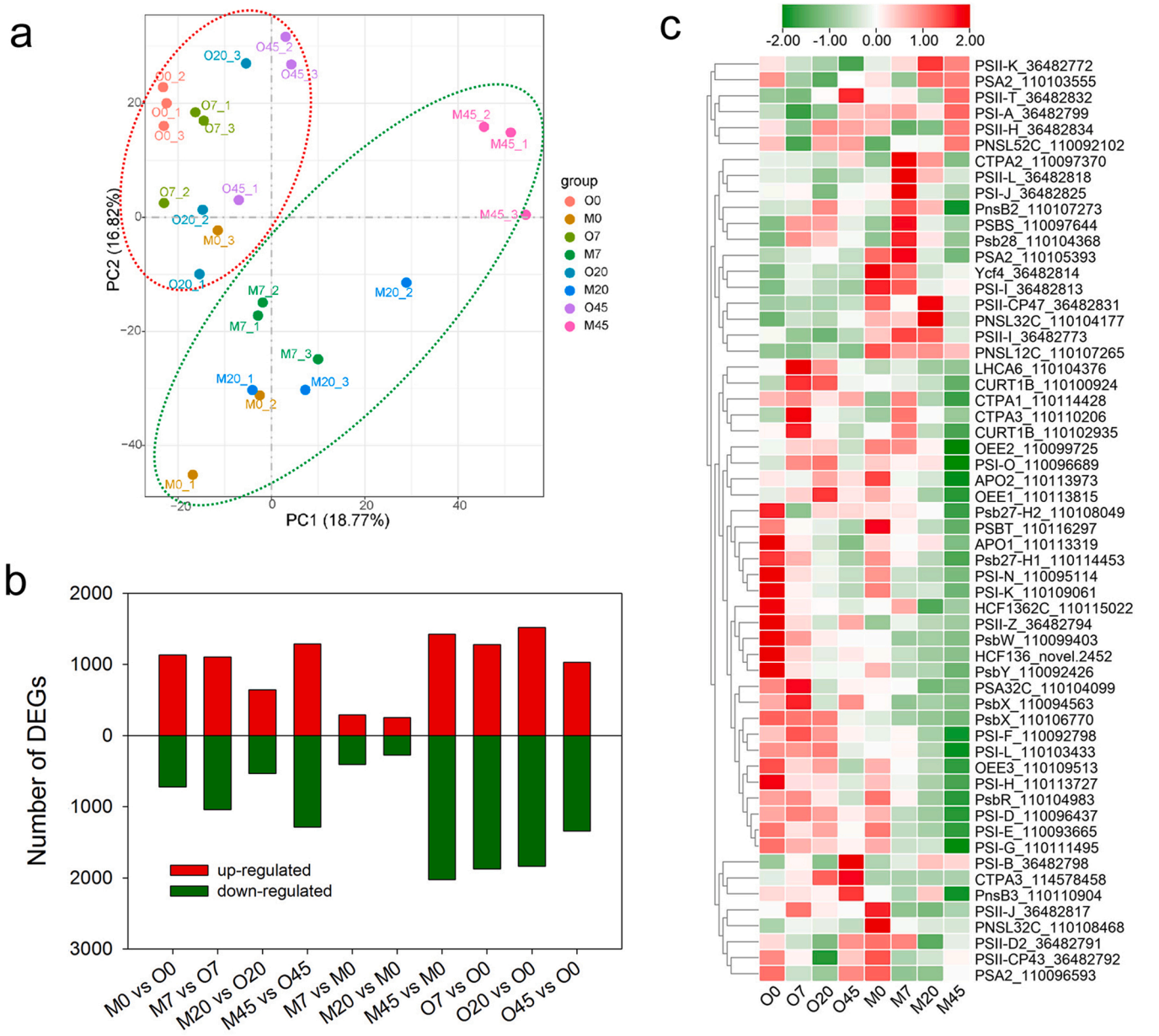

Fig. 3. Global gene expression analysis. a, principal component analysis of all samples. b, the number of differentially expressed genes (DEGs) by pairwise comparison among samples. c, the heatmap of expression levels of genes involved in photosynthesis.

especially in M45 and O45. In M45, many genes were considerably suppressed compared with O45, such as *PSBT* (110116297), *APO1* (110113319), *PSI-N* (110095114), *PSI-K* (110109061), *PsbY* (110092426), and *Psb27-H2* (110108049). These results indicated that drought stress inhibited photosynthesis in both genotypes. Moreover, this phenomenon appeared to be more severe in the M genotype, implying that the M genotype was more susceptible to drought stress than the O genotype.

3.3. Enrichment analysis of DEGs in M and O genotypes under drought

A total of 3835 DEGs were identified in the M genotype under different drought stresses, including 243, 72, and 2863 unique DEGs in M7 vs. M0, M20 vs. M0, and M45 vs. M0, respectively (Fig. 4). KEGG enrichment analysis showed that many genes involved in carotenoid biosynthesis and plant hormone signal transduction were considerably enriched in DEGs between M7 and M0. Genes involved in glycerolipid metabolism and starch and sucrose metabolism were over-represented in DEGs between M20 and M0. Compared with M45 and M0, DEGs were assigned to numerous pathways, such as biosynthesis of secondary metabolites, metabolic pathways, glutathione metabolism, photosynthesis, and amino acids, flavonoid, and unsaturated fatty acids biosynthesis.

A total of 5423 DEGs were identified in the O genotype under different drought stresses, including 1155, 1229, and 642 unique DEGs in O7 vs. O0, O20 vs. O0, and O45 vs. O0, respectively (Fig. 5). By mapping to the KEGG reference pathways, DEGs between O7 and O0 were significantly enriched in many pathways, such as plant hormone signal transduction, mismatch repair, DNA replication, and protein processing in endoplasmic reticulum. It is notable that the plant hormone signal transduction pathway was not only enriched in DEGs sets of O7 vs. O0, but also in M7 vs. M0. Compared with O20 and O0, DEGs were assigned to pathways such as the 2-oxocarboxylic acid metabolism, biosynthesis of amino acids, valine, leucine and isoleucine biosynthesis.

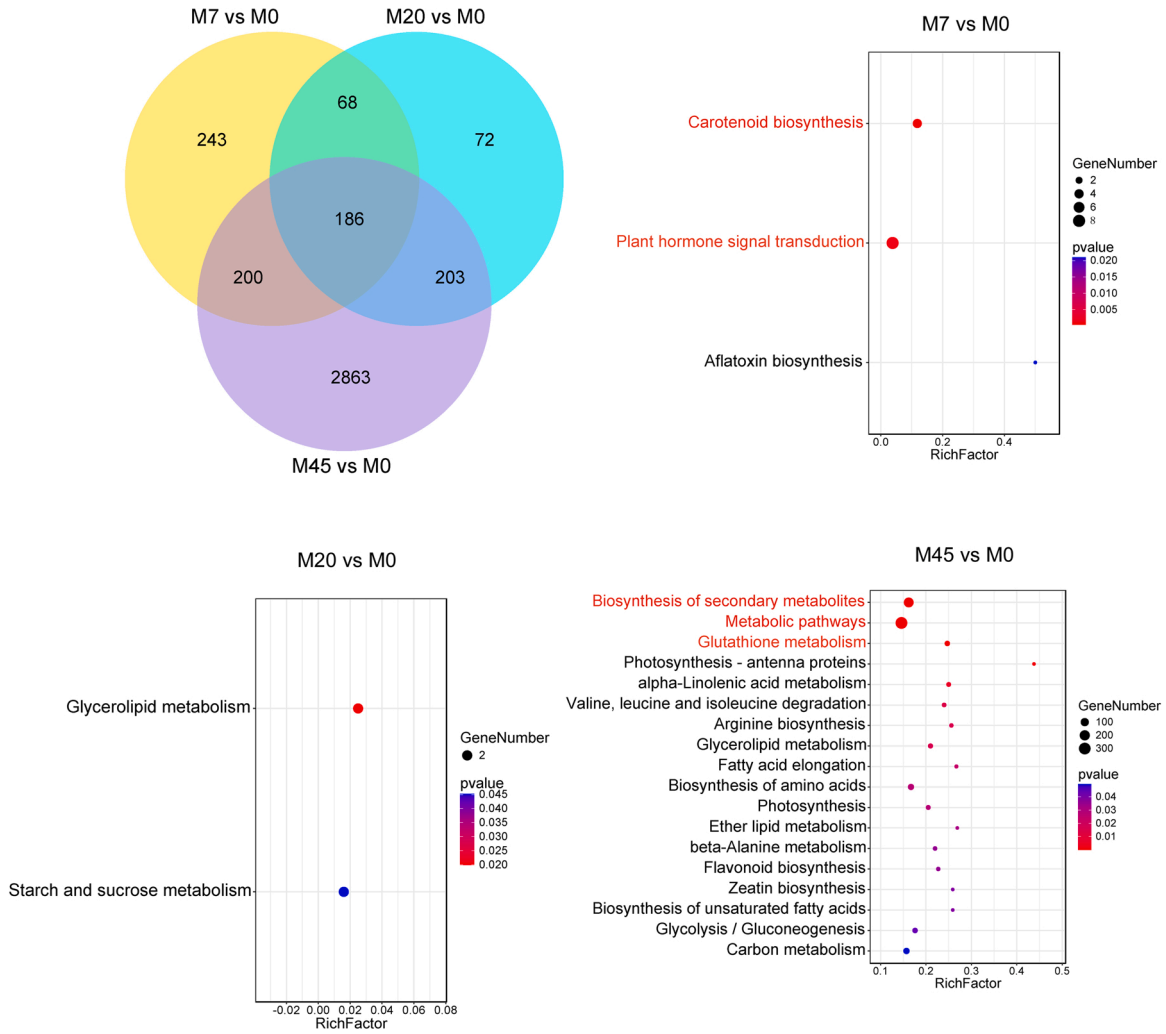

Fig. 4. The venn graph and KEGG enrichment analysis of DEGs among stems of the M genotypes under different drought stress. These enriched pathways with $p < 0.05$ were showed. Of them, these pathways with FDR < 0.05 were shown in red text.

Interestingly, numerous DEGs between O45 and O0 were enriched in the flavonoid biosynthesis pathway similar to the DEGs set between M45 and M0. Moreover, many DEGs were also enriched in phenylpropanoid biosynthesis and flavone and flavonol biosynthesis pathways between O45 vs. O0.

3.4. Enrichment analysis of DEGs between M and O genotype under drought

There were 1106, 1088, 451, and 1808 unique DEGs in M0 vs. O0, M7 vs. O7, M20 vs. O20, and M45 vs. O45, respectively (Fig. 6). Under control condition, many genes involved in the galactose metabolism, glycerolipid metabolism, pentose and glucuronate interconversions, metabolic pathways, phagosome, starch and sucrose metabolism, and DNA replication were differentially expressed between M0 and O0. Under mild drought treatment, the mitogen-activated protein kinase signaling pathway, plant hormone signal transduction; valine, leucine and isoleucine degradation; taurine and hypotaurine metabolism; oxidative phosphorylation; plant-pathogen interaction; tryptophan metabolism; and ribosome biogenesis in eukaryotes were enriched in DEGs between M7 and O7. With increased drought stress (at 20 days), many genes involved in protein processing in the endoplasmic reticulum and sphingolipid metabolism were differentially expressed between M20 and O20. Under severe drought, DEGs were enriched in many pathways, such as citrate cycle, beta-alanine metabolism, carbon metabolism, metabolic pathways, secondary metabolites biosynthesis, alpha-linolenic acid metabolism, and photosynthesis.

3.5. DEGs involved in the plant hormone signal transduction pathway

According to KEGG enrichment analysis, the plant hormone signal transduction pathway plays an important role in the drought response of M and O genotypes under mild drought stress. We reconstructed the pathways involved in ABA biosynthesis and signaling, and BR signaling. In ABA biosynthesis, most identified *PSY*, *PDS*, *ZDS*, *B-CHX*, *ZEP,* and *NCED* genes were up-regulated by drought stress and highly expressed in the two genotype throughout drought treatment or at some specific time points (Fig. 7), except for *ZDS* (110095455), *ZEP* (110103612) and *NCED* (110103406) which were down-regulated under drought in the M genotype. The expression patterns of *CYP707A7* genes, a key enzyme in ABA catabolism, were similar in both genotypes. Two *CYP707A7* genes 110092529 and 110113154 were up-regulated by drought stress. The expression level of *CYP707A7* gene (110095909) was down-regulated by drought in the two genotypes. In the ABA signaling pathway, six of nine *PYR/PYL* were highly expressed in M0. However, two *SnRK2* (110109470, 110114742) were substantially up-regulated in O7 and O20, and were suppressed in M throughout drought treatment. It is notable that 33 of 60 (55%) identified protein phosphatases type 2 C (PP2Cs), a negative regulator of ABA signaling, were significantly up-regulated by severe drought and highly expressed in M45. As a

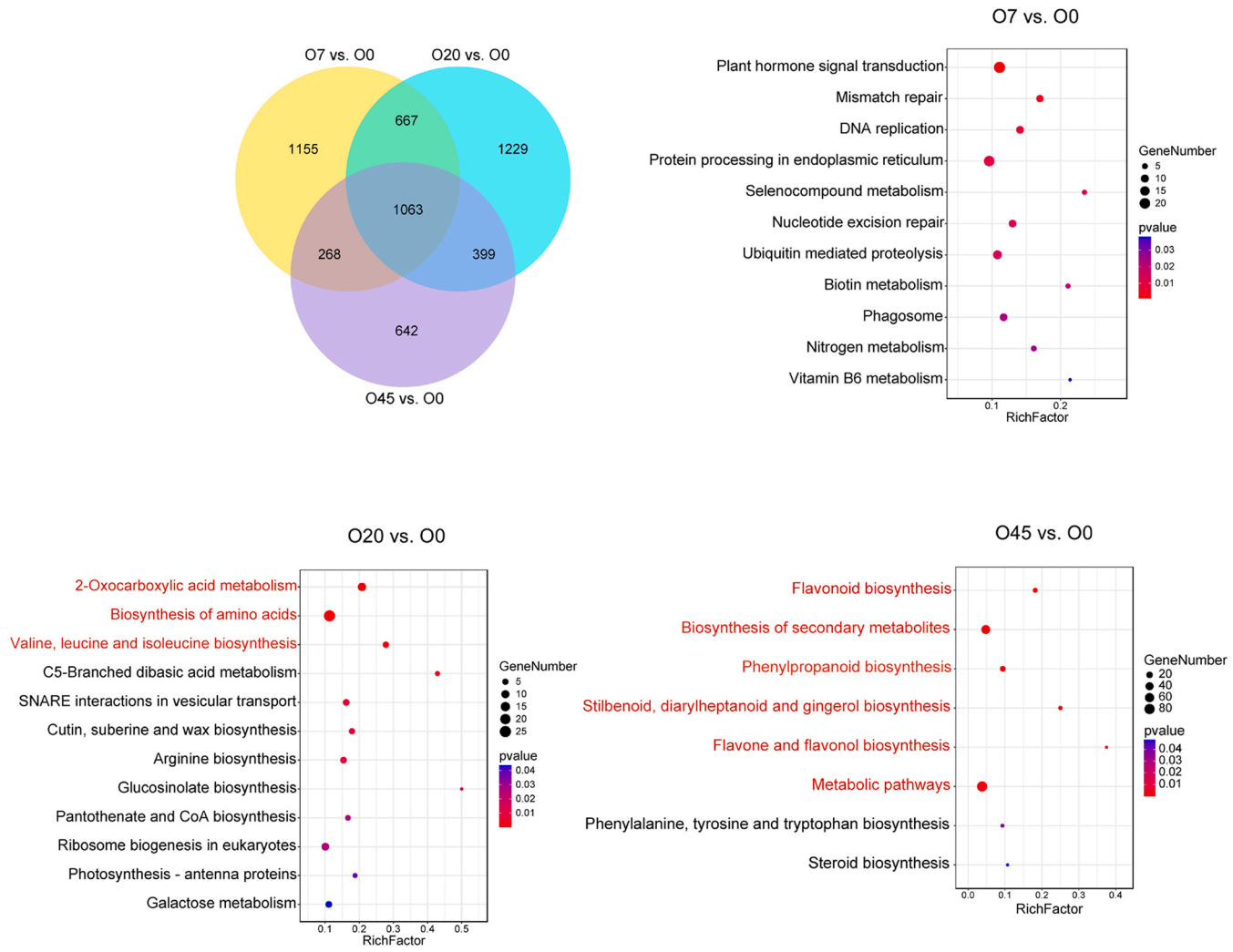

Fig. 5. The venn graph and KEGG enrichment analysis of DEGs among stems of the O genotypes under different drought stress. These enriched pathways with $p < 0.05$ were showed. Of them, these pathways with FDR < 0.05 were shown in red text.

downstream gene of *PP2C*, six *ABI5/ABF* genes were highly expressed in the O genotype, with the exception of 110098225, and four of seven *ABI5/ABF* genes showed higher expression levels in the M genotype.

In the BR signaling pathway, most upstream genes including *BAK1* (110111331 and 110105654), *BRI1* (110103240 and 110115331), *BSK* (110114633 and 110098474), *BSU* (110101617, 110099362, and 110106819), and *BIN2* (11009563) were up-regulated by drought in the M genotype, especially in M45. In contrast, most downstream genes, including *BZR* (110105301, 110102482, 110092501, and 110115742), *TCH4* (110110310,110102810, 110103557, 110115360, 110097938, 114581399, 110108817, 110093423, 110110330, 110109539, 110102443, 110110142, and 110093026), and *CYCD3* (110106939, 110116725, and 110104947) were suppressed by drought in the M genotype. Compared with the M genotype, these genes in the BR signaling pathway were expressed in a less amount in the O genotype (Fig. 7).

The expression patterns of two important protein families LEAs and HSPs involved in drought tolerance of plants were investigated. Most *LEAs* and *HSPs* genes were highly expressed in M45 (15/21, 51.72%) and O0 (36/52, 69.23%), respectively (Fig. 8**a and c**). Five *LEAs* (110098869, 110094501, 110098765, 110115140, and 110102188) genes were up-regulated by mild and moderate drought in the O genotype, and other three *LEAs* (110108899, 110095424, and 110095419) genes showed abundant under severe drought. In the M genotype, most *LEA* genes were up-regulated by drought, especially in M45. Additionally, three DEGs encoding dehydrins (a subset of the LEA proteins) were identified, including 110102734, 110112205, and 110116563 (Fig. 8**b**). All dehydrins were significantly induced by all drought stress conditions, especially 110116563, and reached the peak under severe drought stress in both genotypes. These dehydrins showed higher levels in M45 than in O45. A total of 52 DEGs encoding HSPs were identified. Of them, thirty-six *HSPs* genes showed the most abundant in O0, and forty-one of all *HSPs* (41/52, 78.85%) genes were down-regulated by three drought conditions in the O genotype (Fig. 8**c**). In the M genotype, thirty-five of all *HSPs* (35/52, 67.31%) genes were repressed by drought. Six *HSPs* (110094064, 110115386, 110115835, 110112031, 110110507, and 110104721) were highly expressed in M0 and other seven *HSPs* (110104050, 110106101, 110114126, 110092928, 110105702, 110114122, and 110108419) genes were up-regulated by severe drought in M45 (Fig. 8**c**). Additionally, seven *HSP70* genes levels increased in the M and/or O genotype under different drought stresses (Fig. 8**d**).

3.6. DEGs involved in the flavonoid synthesis pathways

Forty-nine DEGs involved in the flavonoid synthesis pathways were identified, as shown in Fig. 9. Upstream of the pathway, *PAL*, *4CL*, *4CH*, *CHS*, and *CHI* were highly expressed in the O genotype under different drought conditions and at M0. The expression of genes encoding *DFR* (novel.3180, 110093920, 110111528, and 110101655) were high in the O genotype than in the M genotype (Fig. 9**b**). The high expression of

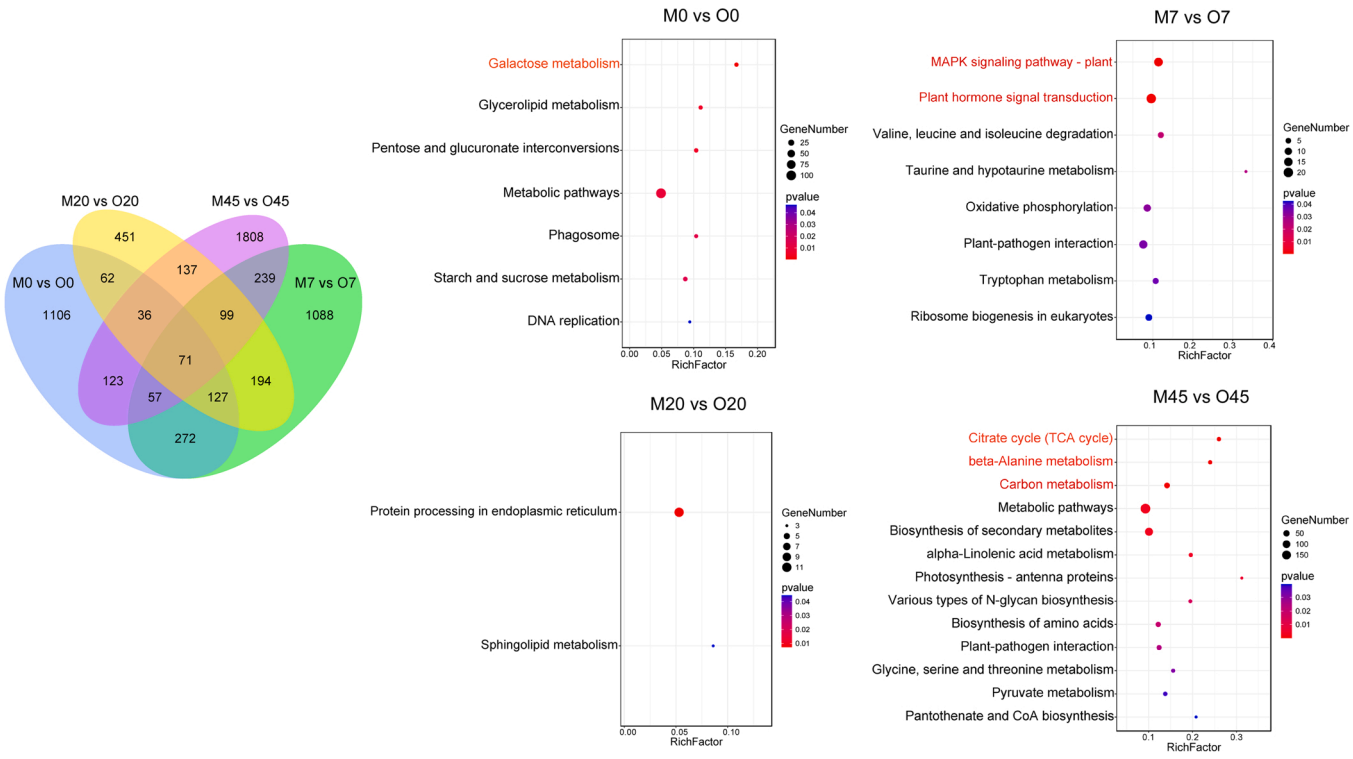

Fig. 6. The venn graph and KEGG enrichment analysis of DEGs between the M and O genotypes under different drought stress. These enriched pathways with $p < 0.05$ were showed. Of them, these pathways with FDR < 0.05 were shown in red text.

those DEGs in the O genotype were consistent with the higher TFC in the O genotype than in the M genotype, especially under control condition and severe drought stress (Fig. 9**c**). However, many downstream genes of flavonoid biosynthesis pathway including *F3′H*, *F3′5′H*, *ANS, UF3GT* were highly expressed in M45.

MYB and basic helix-loop-helix (bHLH) transcription factors (TFs) play important roles in the regulation of flavonoid biosynthesis. We found that MYB (118) is the most abundant TF followed by bHLH (114) and C2H2 (99) (Fig. S1a). The expression analysis showed that most MYBs were highly expressed in M0 and M45. bHLHs were abundant in both genotypes under control condition, and different bHLHs were up- or down-regulated by drought stress in the two genotypes (Fig. S1b and c).

3.7. qRT-PCR analysis of candidate genes

To verify the reliability of the gene expression data generated by RNA-Seq, 15 genes were selected from DEGs set for qRT-PCR analysis.

qRT-PCR analysis demonstrated that these genes exhibited similar up-regulated or down-regulated expression profiles with RNA-Seq analysis (Fig. 10). For instance, the high expression of four HSP genes in O0 including *16.9 kDa HSP*, *26.7 kDa HSP*, *17.1 kDa class II HSP,* and *17.9 kDa class I HSP* were verified by qRT-PCR, which was consistent with the result of RNA-Seq analysis. The results confirmed the validity of the transcriptome data, further verifying the findings from this comparative transcriptome study.

4. Discussion

As one of the most severe natural disasters in the world, drought stress adversely affects plant growth, causes plant death, and reduces crop yields globally. To survive, some plants evolved sophisticated defense mechanisms and possess strong drought tolerance under water deficit stress, such as *D. officinale* which increases its drought tolerance through physical adaptations, environmentally suitable metabolic pathways and molecular regulations (Wan et al., 2018). These coping mechanisms occur in a disciplined spatiotemporal order and are cross-talk, forming a systematic drought response mechanism.

4.1. Different drought tolerance between the two D. officinale genotypes

Drought enhanced inevitably ROS production, provoke oxidation of proteins, lipids, and DNA, as well as plasma membrane damage and death of cells (Lee and Park, 2012). MDA, a membrane lipid peroxide, usually accompanies plant responses to environmental stress, reflecting the extent of membrane peroxidation (Bu et al., 2017). In this work, the MDA content increased rapidly with increasing drought stress in the M genotype, but remained at a steady level in the O genotype under mild (O7) and moderate (O20) drought stress, followed by a significantly increased in O45 under severe drought stress. The results suggest that the effective defense mechanisms in the O type can eliminate the damage caused by ROS under mild and moderate drought stress. However, an apparent oxidative stress occurred in the M genotype under mild and moderate drought because of the fragile defense system. Thus, a relative weaker drought tolerance of the M genotype than that of the O genotype was confirmed by withered leaves and the rapid increase in MDA content under drought conditions. Furthermore, the result was also demonstrated by the more severely repressed expression of photosynthesis-related genes in the M genotype under drought stress, particularly in M45 (Fig. 3**c**). Photosynthesis is the essential source of biomass accumulation for plant growth and development, and is repressed by drought stress which is associated with lower CO_2 concentration as a result of stomatal closure (Xu and Leskovar, 2015; Mo et al., 2016).

Under control condition, the MDA content of drought-tolerant O0 was higher than that of drought-sensitive M0, which might have resulted from different genomic backgrounds of the two genotypes. A similar phenomenon has been reported in two licorice genotypes (Zhang et al., 2022b) and three licorice species (Han et al., 2022). In three licorice species, *Glycyrrhiza uralensis* and *G. glabra* had lower germination

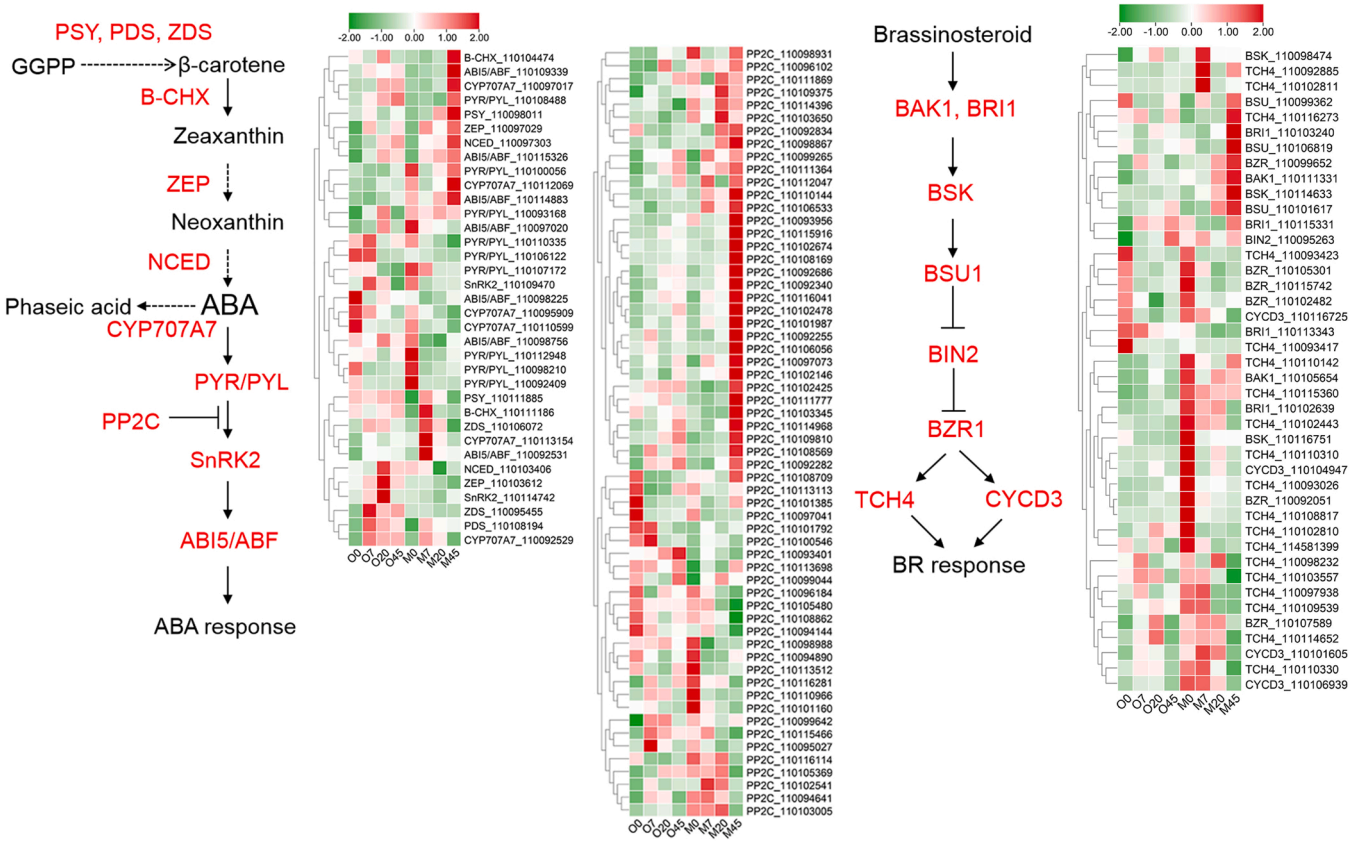

Fig. 7. Biological pathway of ABA biosynthesis and signaling, and BR signaling. PSY: phytoene synthase; PDS: phytoene desaturase; ZDS: zeta-carotene desaturase; B-CHX: beta-carotene 3-hydroxylase; ZEP: zeaxanthin eoxidase; NCED: 9-cis-epoxycarotenoid dioxygenase; CYP707A7: abscisic acid 8′-hydroxylase; PYR/PYL: abscisic acid receptor PYR/PYL family; PP2C: 2 C-type protein phosphatase; SnRK2: sucrose nonfermenting-1-related protein kinase 2; ABI5/ABF: AREB (ABA responsive element binding protein)/ABF (ABRE binding factors); BAK1: BRI1 associated kinase 1; BRI1: brassinosteroid-insensitive 1; BSK: BR-signaling kinase; BSU1: Serine/threonine-protein phosphatase; BIN2: brassinosteroid insensitive 2; BZR1: brassinosteroid resistant 1; TCH4: xyloglucan:xyloglucosyl transferase; CYCD3: cyclin D3.

energy, germination rate, and germination index under water deficit stress comparison with *G. inflata,* Whereas the MDA content of the latter was higher than that of the two former species (Han et al., 2022). Consequently, evaluating drought tolerance based on increasing MDA content under drought stresses, rather than the relative MDA content between genotypes may be more appropriate.

4.2. Different antioxidants to cope with drought stress in the two D. officinale genotypes

Plants have evolved complex protective antioxidant systems to maintain the ROS balance under adverse conditions for survival. The positive correlation between promoted accumulation of enzymatic and non-enzymatic antioxidants and excessive scavenging ROS has been previously reported (Han et al., 2022; Zhang et al., 2022b; Xu et al., 2022). SOD is the first step of defense machinery against ROS in plants for catalyzing the reaction of $O_2^{\bullet -}$ to H_2O_2 (Xu et al., 2011). Our results showed a significant increase in SOD activity in the M genotype under mild and moderate drought stress compared with control plants. However, the SOD level decreased and remained stable levels under different drought stress in the O genotype. Moreover, the SOD levels were significant lower in the O genotype than that in the M genotype under moderate and severe drought. Interestingly, the change patterns of POD activities were similar with that of SOD activities in the both genotypes throughout the treatment. The results suggested that SOD and POD were activated and participated in coping with drought stress in both genotypes, whereas they played a more important role in combating drought stress in the M genotype. The increased SOD and POD activities have positive correlation with drought tolerance in many species (Farooq et al., 2009; Han et al., 2022; Yuan et al., 2022). Drought decreased the SOD activity under drought stress was also observed in previous studies, such as in pea plants at different growth stages (Osman, 2015), pea varieties (Farooq et al., 2021), and *Cerasus humilis* (Ren et al., 2016). Contrary to SOD and POD, CAT and APX activities accumulated gradually with increasing drought stress, and greatly increased under severe drought stress in both genotypes (Figs. 2**d and** 2**f**). CAT activity was significant higher in the O genotype than in the M genotype throughout the treatment, except for under mild drought. Furthermore, APX activity showed significant higher levels in the O genotype than that in the M genotype under moderate and severe drought (Fig. 2**f**). CAT and APX are involved in decomposing the H_2O_2 generated by SOD into water and molecular oxygen (Reddy et al., 2004). A notable high CAT and APX activity were also found in drought-tolerant maize seeds (Huang, Song, 2013) and tobacco plants treated by spermidine compared with control plants under drought stress (Xu et al., 2022). CAT and APX may play more important role in drought tolerance of *D. officinale.* Accordingly, we hypothesize that the M and O genotypes employed different antioxidant enzymes to scavenge excessive ROS under drought stress. Additionally, proline levels were significantly increased by drought in both genotypes and may also contribute to scavenge excessive ROS, especially in the O genotype with higher proline contents during drought treatment. The accumulation of proline in dehydrated plants could serve as a potent non-enzymatic antioxidant to scavenge ROS (Ben Rejeb et al., 2014).

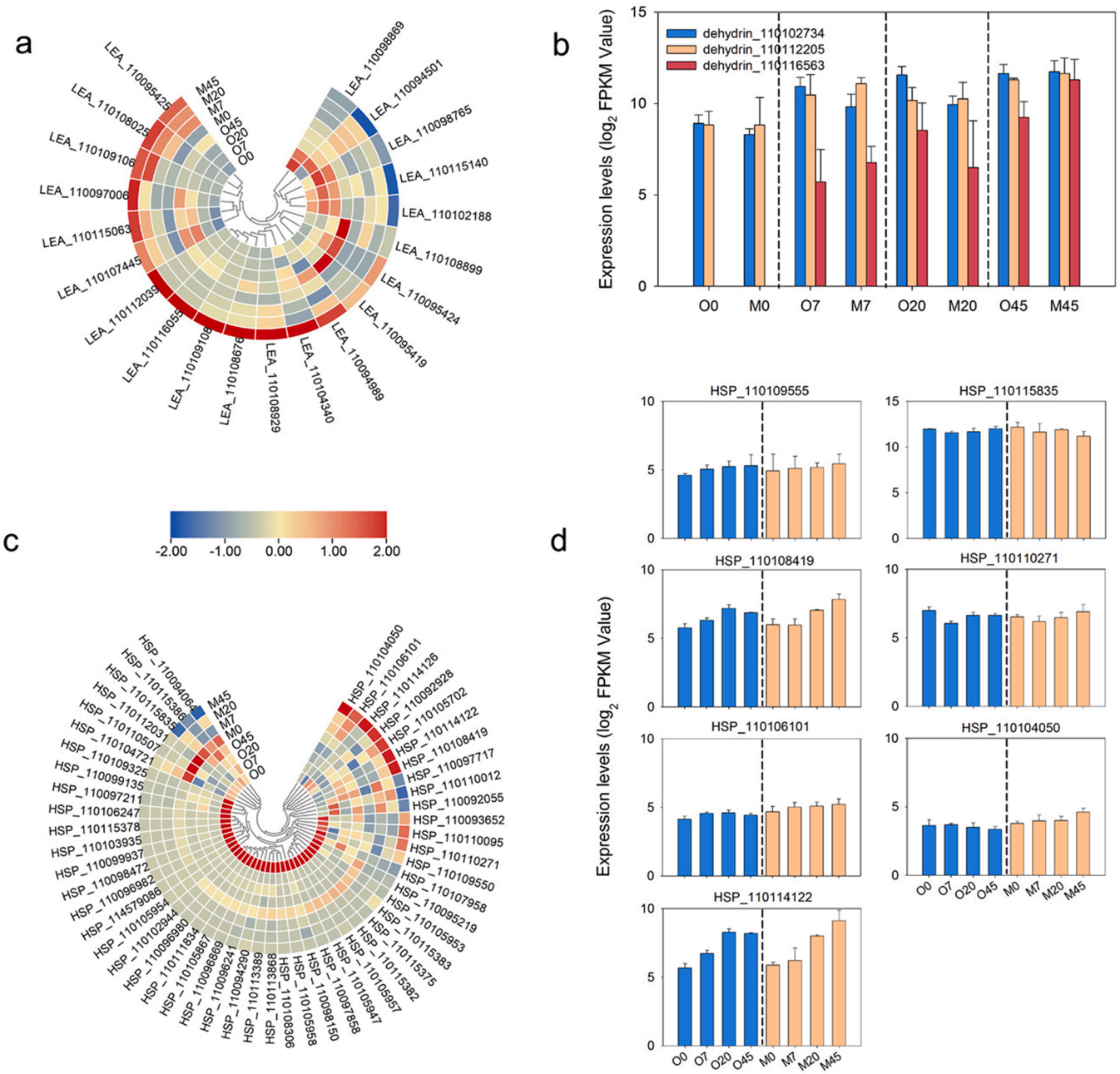

Fig. 8. The expression level of *LEAs* and *HSPs* genes in the two *D. officinale* genotypes under different drought stress. a, the expression levels of *LEAs*. b, the expression levels (log_2 FPKM Value) of dehydrins. **c**, the expression levels of *HSPs*. **d**, the expression levels (log_2 FPKM Value) of *HSP70*.

4.3. A significant role of plant hormone signal transduction pathway in the different drought tolerance of the two D. officinale genotypes

Drought stress triggered different transcriptional reprogramming between the two *D. officinale* genotypes under different drought stress conditions. Under mild drought stress (drought for 7 days), many DEGs involved in the plant hormone signal transduction were enriched consistently in DEG sets of M7 vs. M0, O7 vs. O0, and M7 vs. O7, indicating its important role in the response of *D. officinale* to mild drought stress. Huang et al. (2021) showed a significant positive correlation between plant hormone biosynthesis and signal transduction with facultative CAM, especially ABA. ABA accumulates after drought stress, and is an essential factor that positively regulates plant drought-stress responses (Sato et al., 2018). In our study, we found that more DEGs involved in ABA biosynthesis and signaling pathway were up-regulated by severe drought stress in M45 than in O45. Remarkably, many PP2Cs were significantly induced by severe drought stress in M45 but less expressed in O45 (Fig. 6). The PP2Cs is key negative regulator of ABA signaling via interacting and inhibiting the kinase activity of the positive regulator SnRK2s (Zhang et al., 2013). Chen et al. (2021) indicated that the GhDRP1 (a member of PP2Cs) overexpression transgenic cotton showed reduced drought tolerance, contrary to the GhDRP1-silenced (RNAi) cotton. The elimination of Group A PP2C is sufficient to ensure *Physcomitrella patens* survival to full desiccation, without ABA treatment (Komatus et al., 2013). The recessive loss-of-function mutant *hab1–1,* a member of PP2Cs from group A, shows ABA hypersensitive inhibition of seed germination and enhanced ABA-mediated stomatal closure (Kuhn et al., 2006). So, we speculated that the high expression of many PP2Cs in M45 resulted in its ABA hyposensitive compared with O45, leading to a relative weaker drought tolerance of the M genotype under drought

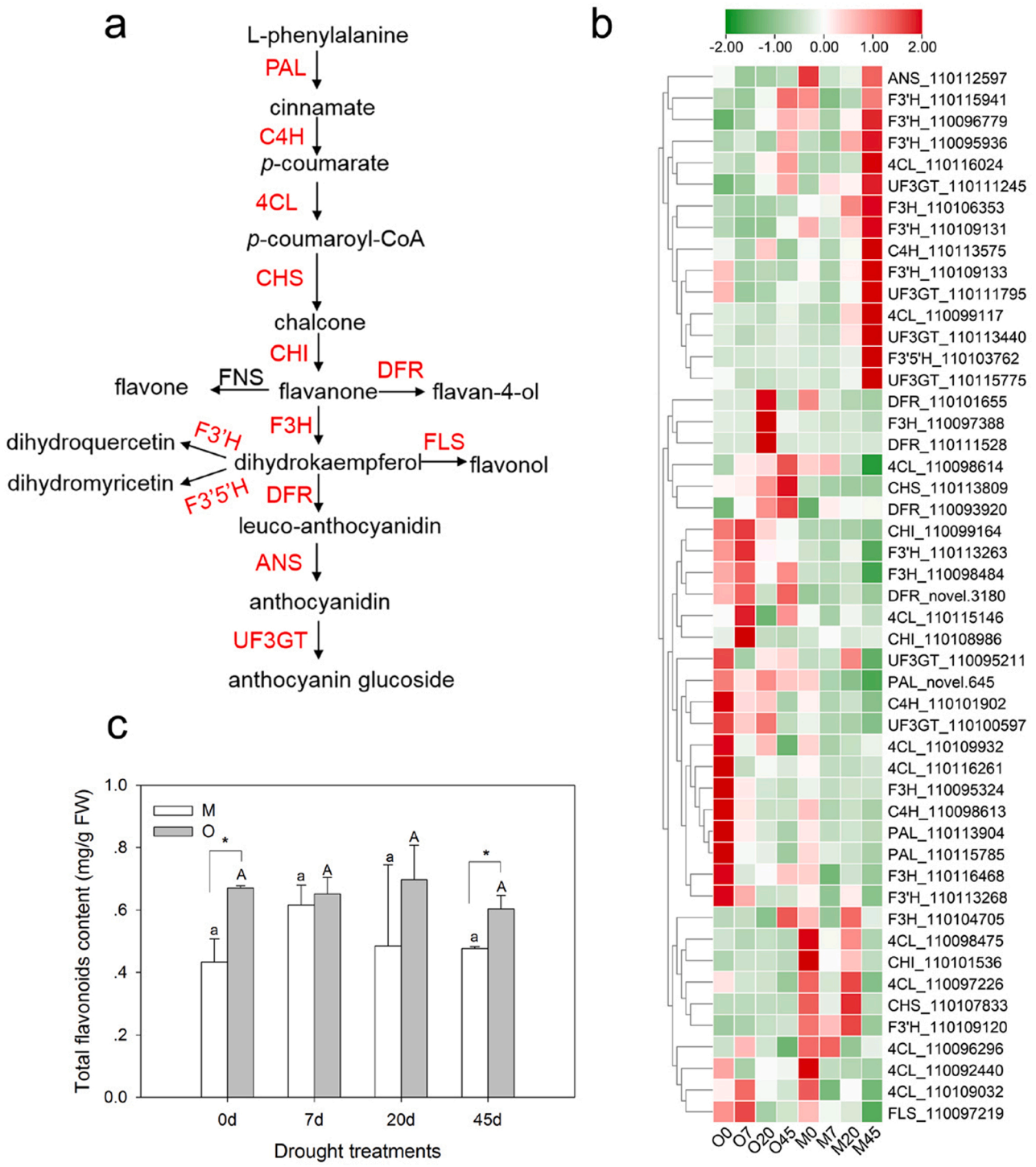

Fig. 9. Biological pathway of flavonoid biosynthesis and the total flavonoid contents. a, the flavonoid biosynthesis pathway. b, the expression patterns of DEGs involved in flavonoid biosynthesis pathway. c, the total flavonoid contents. PAL: phenylalanine ammonia-lyase; C4H: trans-cinnamate 4-monooxygenase; 4CL: 4-coumarate CoA ligase; CHS: chalcone synthase; CHI: chalcone isomerase; F3H, flavanone 3-dioxygenase; DFR: dihydroflavonol 4-reductase; FLS: flavonol synthase; F3'5'H: flavonoid 3′5'-hydroxylase; F3'H: Flavonoid 3′-hydroxylase; ANS: anthocyanidin synthase; UF3GT: UDP-glucose flavonoid 3-O-glucosyltransferase.

stress. Moreover, the low expression levels of *SnRK2s* in the M genotype also corroborated the inhibitory effect of highly expressed PP2Cs on the kinase activity of *SnRK2s*. Clearly more information is needed on the function and regulation of PP2C on drought tolerance in *D. officinale*.

Complex antagonistic interactions between ABA and BR signaling pathways have been documented. ABA represses *A. thaliana* seed germination and postgerminative growth, whereas BRs antagonize ABA-mediated inhibition and promote these processes (Hu and Yu, 2014). BRs can suppress the ABA signaling during early seedling development by activating BES1-TPL-HAD19 repressor complex which controls epigenetic silencing of *ABI3* (Ryu et al., 2014). In our study, many genes involved in the BR signaling pathway were highly expressed in M0, and induced by drought stress in the M genotype, especially in M45. These genes might antagonize ABA-mediated drought tolerance in the M genotype. In *Arabidopsis*, BRINSENSITIVE1 (BRI1)-EMS-SUPPRESSOR1 (BES1), a transcription factor of the BR signaling pathway, directly and

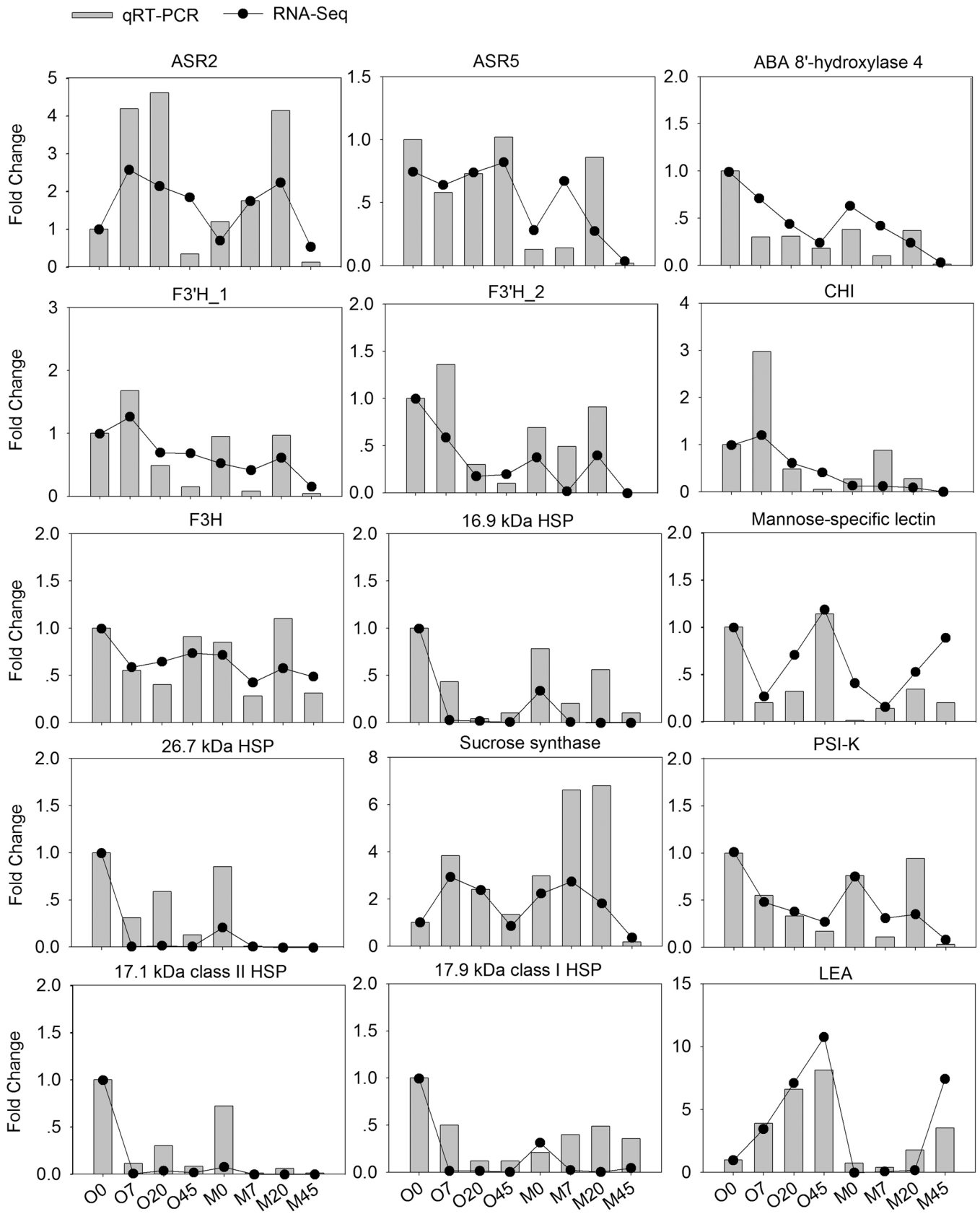

Fig. 10. qRT-PCR validation of candidate genes. Error bars represent ± SD of triplicates for qRT-PCR.

indirectly suppresses the transcriptional activity of ABI5, and consequently reduces ABA signaling output (Ryu et al., 2014; Zhao et al., 2018). Phytohormone crosstalk is an important adaptive mechanism existed in plants to balance growth and survival under adverse conditions.

LEAs, dehydrins and HSPs, downstream to ABA, have been reported to regulate various abiotic stress responses, including drought, heat, cold, and salinity (Manfre et al., 2006; Kumar et al., 2012; Liu et al., 2015). Under drought stress, the activity of LEAs is up-regulated to prevent target proteins from denaturation and aggregation (Manfre et al., 2006). In the two *D. officinale* genotypes, most *LEAs* genes, including three dehydrins, were present in less quantities in O0 and M0 under control condition, and were up-regulated by drought, showing the highest abundance in M45 (Fig. 7). In foxtail millet, overexpression of *SiLEA14* provided high tolerance to drought and high osmolarity (Wang et al., 2014a). Overexpression of dehydrin genes improve drought tolerance, which has been also confirmed in many species (Xiao et al., 2007; Xie et al., 2012; Kumar et al., 2014). Thus, we infer that LEAs may play an important role in response to drought stress of *D. officinale,* particularly in M genotype. Intriguingly, in contrast to LEAs, the expression of forty-one (78.85% of all *HSPs* genes) *HSPs* genes in O0 and thirty-five (67.31%) *HSPs* genes in M0 were considerably repressed by drought (Fig. 7). HSPs act as molecular chaperone through their role in perpetuating cellular stability and protecting cell against a wide variety of stress (Wang et al., 2014b). A recent study reported that drought stress could dissociate the MdHSP90-MdHSFA8a complex which inhibits the latter binding activity and transcriptional activation under control condition, and the released HSFs activated the expression of downstream drought-responsive genes to promote apple (*Malus domestica*) survival during drought (Wang et al., 2020). The significantly decreasing expression of *HSPs* under different drought stress, particularly in the O genotype, might be related to the releasing the HSF and increased ABA signaling, and then promote drought tolerance of *D. officinale.* In addition, several *HSP70* genes were induced by drought stress in the O or/and M genotype (Fig. 7d), suggesting their potential functions in conferring drought tolerance upon *D. officinale*. Overexpressing heat-shock protein improves drought tolerance in rice (Xiang et al., 2018), as well as in tomato (Aghaie, Tafreshi, 2020) and pepper (Feng et al., 2019), which was attributed to the hypersensitive to ABA of the transcription of ABA-responsive genes (Clément et al., 2011). This study shed a light on the differential regulation of *LEAs* and *HSPs* genes in response to drought in the two *D. officinale* genotypes suggested variations in the role of these proteins in drought tolerance.

4.4. Severe drought induced the flavonoid biosynthesis pathway in the two D. officinale genotypes

Under drought stress, plant cells have evolved a network of enzymatic and nonenzymatic antioxidant mechanisms to maintain ROS homeostasis and prevent oxidative stress (Wrzaczek et al., 2013). Flavonoids are vital plant secondary metabolites and strong antioxidant that provide protection to plants from abiotic and biotic stresses (Treutter, 2006; Nakabayashi et al., 2014; Shojaie et al., 2016). In our study, the TFC was evidently higher in stems of the O genotype than that of the M genotype under control and drought stress conditions (Fig. 8). It has been postulated that abundant flavonoids participate in the scavenging of oxygen free radicals, mitigate against oxidative and improve drought tolerance of the O genotype under severe drought stress, as reported in *A. thaliana* and maize (Nakabayashi et al., 2014; Li et al., 2021). The most flavonoid biosynthesis genes encoding *PAL*, *C4H*, *4CL*, *CHS*, *CHI,* and *DFR* were also highly expressed in the O genotype, especially *PAL* and *DFR*. PAL is a key enzyme that catalyzed the conversion of L-phenylalanine to trans-cinnamic acid to supply precursor for the lignin and flavonoid biosynthetic pathways (Bagal et al., 2012). DFR is a key enzyme in the biosynthesis of anthocyanidins, proanthocyanidins, and other flavonoids (Winkel-Shirley, 2002). These highly expressed flavonoid biosynthesis genes might result into the higher TFC in the O genotype than in the M genotype, which maintain ROS homeostasis and prevent oxidative stress, and thereby conferred stronger drought tolerance to the O genotype. Drought stress increase the amount of flavonoids in wheat (Ma et al., 2014). However, the total flavonoids accumulation were accelerated firstly, and then slightly decreased in the stems of both *D. officinale* genotypes in our study, which were similar to the results of Shojaie et al. (2016). Under severe drought stress, flavonoid 3-hydroxylase (F3′H) and flavonoid 3′5′-hydroxylase (F3′5′H) were up-regulated in both of *D. officinale* genotypes. The two key enzymes control the hydroxylation pattern of the flavonoid B-ring and catalyze the biosynthesis of three different anthocyanins, leading to the great diversification of the flavonoid biosynthesis pathway (Tanaka and Brugliera, 2013). This metabolic diversification has been suggested to provide the mechanism for biochemical adaptation in plant defense under diverse environmental conditions during evolution. Indeed, flavonoids may accomplish their protective role by both scavenging of oxygen free radicals and regulating stomatal switches as an ABA-dependent manner under drought stress (Watkins et al., 2017). The facultative CAM is the drought-induced photosynthetic transitions between C_3 and CAM with an inverse day/night pattern of stomatal opening, which has been found in *D. officinale* (Su, Zhang, 2003). It is important to further detect the role and regulation mechanism of flavonoids in facultative CAM of *D. officinale* under drought stress.

As we know, drought-tolerance strategy in plants is a complex scheme involving a finely regulated network of communication among various signals. We investigated several antioxidants levels and gene expression pattern of two *D. officinale* under different drought stress in this study. However, it is still difficult to make universal generalization about the regulation mechanism underlying drought-tolerance of *D. officinale.* A more comprehensive investigation of the ROS scavenging system and metabolome will be beneficial for fully revealing the regulatory mechanism of drought resistance in *D. officinale.* Additionally, the biochemical, gene expression and metabolites details of CAM is a very promising direction to reveal the regulatory mechanism of drought resistance in *D. officinale*.

5. Conclusion

In present study, we comprehensively analyzed the biochemical and transcriptomic profiles of two *D. officinale* genotypes under three drought stress conditions. The O genotype demonstrated a better defense against drought stress, by limited MDA accumulation, a stranger CAT and APX activity, effective ABA signaling pathway, and a higher flavonoid content. Contrary to the O genotype, the M genotype showed a relative weaker drought tolerance, with repressed photosynthesis-related genes, sharply increased MDA content, a lower CAT and APX activity, highly expressed PP2Cs, and a lower flavonoid content. This study shed new light on the coordination of multiple signaling pathways in regulation drought tolerance of *D. officinale.* In summary, our results might assist breed crops adapted to increasing drought stress.

CRediT authorship contribution statement

Hui Huang: Conceptualization, Methodology, Project administration, Data curation, Software, Writing – review & editing. **Yixue Jiao:** Writing – original draft. **Yan Tong**: Acquired the data. **Yuhua Wang:** Conceptualization, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.indcrop.2023.116766.

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