

Transcriptomics-proteomics analysis reveals the role of SiNRX1 in regulating drought stress in foxtail millet (*Setaria italica* L.)

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Abstract

Nucleoredoxin (NRX), a member of the thioredoxin (TRX) superfamily, is involved in regulating plant growth and development, as well as abiotic and biotic stress processes, but it is not clear whether NRX participates in regulating the drought resistance of foxtail millet. In this study, the *SiNRX1* of foxtail millet was knocked out using CRISPR/Cas9 system, and the drought resistance of *sinrx1* mutants was identified at both the germination stage and the seedling stage. Moreover, through transcriptome sequencing and data-independent acquisition (DIA) quantitative proteomics determination of *sinrx1* mutants and wild types (WT) at the seedling stage under drought and control conditions, the molecular mechanism of *SiNRX1* regulating drought resistance was preliminarily analyzed. The results indicated that after 7 days of simulated drought treatment during the germination period, the germination rate, root length and bud length of the *sinrx1* mutant decreased significantly in comparison with the WT. During the seedling stage under drought stress, the survival rate, chlorophyll content, proline content, peroxidase (POD) activity and catalase (CAT) activity of the *sinrx1* mutant decreased markedly compared to those of the WT plants. However, the malondialdehyde (MDA) content of the *sinrx1* mutant was significantly higher than that of the WT. This indicates that, during the germination and seedling stages, the drought resistance of the *sinrx1* mutant decreased significantly compared to that of the WT. The transcriptome findings suggested that the 2253 differentially expressed genes (DEGs) (1179 up-regulated DEGs and 1074 down-regulated DEGs) are drought-responsive genes specifically influenced by *SiNRX1*. The proteomic results suggests that the 155 differentially expressed proteins (DEPs) (127 up-regulated DEPs and 28 down-regulated DEPs) are drought-responsive proteins specifically influenced by *SiNRX1*. Moreover, 2253 DEGs and 155 DEPs were significantly and jointly enriched in the phenylpropanoid biosynthesis pathway. This study offers theoretical guidance for the analysis of the drought resistance mechanism of foxtail millet plants and for drought resistance breeding.

1. Introduction

Global warming has intensified droughts in different regions worldwide to varying degrees, but drought, an abiotic stress, limits agricultural production and poses a threat to food security. Hence, it is particularly crucial to identify drought resistance genes, analyze the drought resistance mechanism of drought-tolerant crops and undertake drought resistance breeding [1–4]. When plants are exposed to drought stress, it may lead to the accumulation of reactive oxygen species (ROS), which in turn causes damage to biological macromolecules in plants and restrains their growth [5–7]. To prevent ROS imbalance within the body, plants have evolved an efficient antioxidant defense system to eliminate ROS, thereby enabling plants to possess a certain resistance to abiotic stress [8–10]. The analysis of the function and pathway of the proteins involved in the antioxidant defense system has certain guiding significance for drought resistance breeding.

Thioredoxin (TRX), featuring a thiol-disulfide active site, participates in the oxidoreductase system and regulates ROS [11–15]. Based on the active sites, TRX is classified into typical TRX (types f, h, m, o, x, y, and z) and atypical TRX (such as NRX, CDSP32, NTRC, etc.) [3, 16–19]. The NADPH-dependent TRX

reductase A (NTRA) and B (NTRB), as well as mitochondrial thioredoxin o1 (TRX o1) in *Arabidopsis thaliana*, are implicated in regulating the drought resistance via metabolic pathways [20]. In watermelon (*Citrullus lanatus*), CITRX h2 interacts with CIMPCK5 to inhibit the phosphorylation of CIMPCK3, thereby regulating the CBF-COR signaling pathway under low temperature stress and establishing a connection between TRX and the MAPK cascade signaling [21]. TRX h2 responds to aluminum stress and regulates leaf senescence through the ABA-mediated ROS pathway in *Arabidopsis thaliana* [22]. TRX h5 promotes the expression of immune genes induced by salicylic acid (SA) by regulating the conformational state of the SA response coactivator NPR1 [23, 24]. TRX regulates the levels of ROS or affects plant pathways by modulating the activity of related proteins, thereby regulating plant growth and responding to biological or abiotic stresses.

Nucleoredoxin (NRX), a member of the TRX superfamily, possesses multiple TRX folding and active sites, which might enable NRX to interact with various substrates and NRX has the redox capacity characteristic of TRX [24–26]. The independent TRX-like domain in ZmNRX possesses redox activity and plays a significant role in cell growth and differentiation [27]. Two types of NRX (*AtNRX1* and *AtNRX2*) were identified in *Arabidopsis*, and the mutant of *atnrx1* exhibited reduced pollen fertility. Moreover, *AtNRX1* regulates catalase activity, mediates the antioxidant enzyme system, and safeguards plant cells against oxidative stress [25, 26]. *AtNRX2* functions in the formation of *Arabidopsis* trichomes by mediating the jasminate (JA) hormone pathway [28]. In plants, NRX can interact with phosphofructokinase 1 (PFK1) to regulate the activity of PFK1, thereby maintaining a balance between glycolysis and the pentose phosphate pathway [29]. Overexpression of *TaNRX1* in wheat improved the drought resistance of transgenic wheat [30]. *SiNRX1* regulates heat tolerance by enhancing antioxidant capacity and inducing heat shock proteins (HSPs), and promotes Psm (*Pseudomonas syringae* pv. Maculicola) pathogen infection through interfering with the salicylic acid (SA) hormone signaling pathway of the plant and increasing the accumulation of H₂O₂ content in tomato [31, 32]. *SiNRX1*, *SiNRX2* and *SiNRX3* were identified in foxtail millet and the overexpression of *SiNRX1* in *Arabidopsis* enhanced the drought and salt tolerance of transgenic *Arabidopsis* [3]. NRX mediates the redox system, the hormone transduction pathway and other pathways in vivo to regulate plant growth and development and respond to abiotic and biotic stress.

Foxtail millet (*Setaria italica* L.) is a C4 model crop with a high-quality reference genome sequence, which provides a reliable genomic resource for the study of foxtail millet's stress resistance and can serve as an alternative genome for the research and improvement of switchgrass (*Panicum virgatum*) and related biofuel crops [33]. Moreover, foxtail millet serves as a major source of food and feed in certain parts of Asia and Africa. Nevertheless, due to environmental changes, drought has emerged as one of the main factors restricting the production of foxtail millet [34–36]. Studying the drought resistance regulation mechanism of foxtail millet is of great significance for analyzing the drought resistance mechanism of C4 crops and for drought resistance breeding [4, 37–39]. NRX is a member of the TRX superfamily, which possesses TRX enzyme activity and might maintain the redox balance in plants. The overexpression of *SiNRX1* in *Arabidopsis* enhanced the tolerance of transgenic *Arabidopsis* plants to drought stress; however, the regulatory pattern of *SiNRX1* in response to drought stress

requires further investigation [3]. In this study, the CRISPR/Cas9 system was utilized to knockout the *SiNRX1* gene in foxtail millet, and to determine the drought resistance of *sinrx1* mutant lines at the germination and seedling stages. The regulation of *SiNRX1* in response to drought stress was analyzed through RNA-seq and data-independent acquisition (DIA) quantitative proteomics of samples under normal treatment and drought stress. The objective of this research is to gain a profound understanding of the drought-resistance mechanisms in foxtail millet plants and offer theoretical guidance for drought-resistance breeding endeavors.

2. Materials and methods

2.1 Acquisition of mutants of *sinrx1* from foxtail millet

To verify the drought resistance of *SiNRX1* in foxtail millet, the CRISPR/Cas9-mediated gene editing system was employed to knockout the *SiNRX1* gene in foxtail millet [40]. The *sinrx1* mutant was acquired through genetic transformation by using the 20 bp sequence of single guide RNA (sgRNA) with the original spacer adjacent motif (PAM) on exon 2 of *SiNRX1* as the target. The receptor of the *sinrx1* mutant is Jingu 21 (Jin21; WT). Jingu 21 is a high-quality foxtail millet variety developed by the Economic Crops Research Institute of Shanxi Academy of Agricultural Sciences through continuous single-plant selection after irradiating the strain Jin Fen 52 with cobalt-60 gamma rays. This variety was approved by the Ministry of Agriculture and Rural Affairs of the People's Republic of China in 1991 and has been widely planted as a main multigrain crop due to its wide adaptability and good stability in the arid and semi-arid regions of northwest China (including Shanxi, Shaanxi and other provinces) [41–44]. In addition, the DNA containing the target sequence was amplified from the leaves of the transgenic plants. After gel recovery and purification, the PCR products were transferred into the pEASY-T₁ cloning vector (Vazyme, Nanjing, China) and transformed into DH5 α competent cells (Tsingke, Beijing, China) through the heat shock method. Subsequently, the samples were sent to Beijing Tsingke Biotechnology Co., Ltd (<https://www.tsingke.com.cn/>) for sequencing and were compared with the *SiNRX1* sequence to identify the editing type. The specific primers are shown in Table S1.

2.2 The growth conditions of the wild type (WT) and *sinrx1* mutants of foxtail millet.

Seeds of WT and the T₄ generation of *sinrx1* mutant were sterilized with 1% NaClO and placed in nutrient soil (soil: vermiculite = 3:1, v/v) and incubators (temperature 28 °C/22 °C, light 15 h/dark 9 h, humidity 60–70%, light intensity 17000 lux). The WT and *sinrx1* mutants were planted in the same pot to control environmental effects. The control (CK) and drought treatment (DS) groups were established. Each group consists of 24 pots as one repetition, and there are three replicates for each experiment. When the foxtail millet reached the three-leaf stage, the control group (CK) was watered normally, while the drought treatment group (DS) ceased watering until the foxtail millet phenotype emerged, in order to identify the drought resistance of the WT and *sinrx1* mutant seedlings at this stage. When the plants in the drought treatment group were exposed to drought stress for 8 days and the leaves of the plants

presented a wilting state, the leaf materials of foxtail millet were collected and stored at -80 °C for RNA-seq analysis, proteome analysis and physiological parameter estimation.

2.3 Estimation of biochemical parameters

To estimate various biochemical parameters under drought stress, it was categorized into the germination stage and the seedling stage. According to the inspection of grain and oils-germination test of seeds (GB/T 5520 – 2011), the identification of simulated drought in foxtail millet at the germination stage was carried out. 100 seeds WT and 100 seeds of *sinrx1* mutants were disinfected with 1% NaClO, followed by 20% PEG 6000 osmotic simulated drought treatment, with distilled water treatment being used as the control. Biological repetition was conducted three times. The germination rate, root length, and bud length were measured after 7 days of dark treatment at a temperature of 25 °C/20 °C. The biochemical parameters of the seedling stage were evaluated by culturing the WT and *sinrx1* mutants under the drought treatment of three-leaf stage. Subsequently, the drought tolerance indexes of CK and DS were measured, including chlorophyll content, proline content, malonaldehyde (MDA) content, peroxidase (POD) activity and catalase (CAT) activity. The chlorophyll content of foxtail millet leaves in the same part was determined using the SPAD-502 chlorophyll analyzer, with 15 biological replicates and three technical replicates carried out. The contents of proline (G0111W), MDA (G0109W), POD (G0107W) and CAT (G0105W) were determined by using the microplate assay kit of Suzhou Gris Biological Company (<http://www.gerui-si-bio.com/>). Three biological replicates and three technical replicates were performed.

2.4 RNA-seq and data analyses

The leaves of *sinrx1* mutants (M-3) and Jingu 21 (WT; Jin21) plants were collected following normal watering and drought treatment, and their transcriptomes were analyzed through RNA-seq analysis. The samples from every three plants were combined into one independent sample, and the three independent samples were aggregated for analysis. The RNA library preparation and sequencing were accomplished by Novogene Co., Ltd (Beijing, China). Employ the Agilent 2100 bioanalyzer (Agilent Technologies, CA, USA) to precisely determine the integrity and total quantity of RNA. Once the library test is passed, different libraries are combined in accordance with the requirements of effective concentration and target data volume, followed by Illumina sequencing, generating 150 bp paired end reads. The fundamental principle of sequencing is sequencing by synthesis. Clean reads were obtained by processing raw reads with fastp software and eliminating reads containing adapter, reads containing N and low-quality reads. Clean reads were then mapped to the foxtail millet reference genome (https://phytozome-next.jgi.doe.gov/info/Sitalica_v2_2) using HISAT2 v2.0.5 software [45].

The new gene was predicted by StringTie (1.3.3b) and FPKM was calculated with featureCounts (1.5.0-p3) [46, 47]. The differentially expressed genes (DEGs) between the two comparative combinations was analyzed using DESeq2 software (1.20.0) [48]. $|\log_2(\text{FoldChange})| > 1$ & $\text{padj} < 0.05$ were used as the screening criteria. GO enrichment and KEGG enrichment analysis of DEGs were conducted by

clusterProfiler (3.8.1) software [49]. All normalized cluster analysis and correlation analysis were carried out using R (Version 3.5.0) software.

2.5 Reverse transcription quantitative PCR (RT-qPCR) validation

To verify the reliability of the transcriptome, RT-qPCR was employed to verify the expression levels of 8 genes. Using the total RNA extraction kit from Beijing Zoman Biotechnology Co., Ltd (<http://zomanbio.com/>), with product number ZP402, RNA is extracted from the leaves. The first strand cDNA was synthesized through reverse transcription with the EX RT kit (gDNA remover) of Beijing Zoman Biotechnology Co., Ltd, article number ZR-108. According to the manufacturer's instructions, RT-qPCR was performed on the QuantStudio 3 real-time PCR system using 2×HQ SYBR qPCR Mix (No.: ZF502) of Beijing Zoman Biotechnology Co., Ltd. The expression levels of the genes were subsequently calculated based on $-\Delta\Delta C_t$. All samples were processed with at least three biological replicates and three technical replicates, and SiActin-7 (ID: Seita.5G393000) was employed as an internal reference primer; the specific primers utilized above are presented in Table S1.

2.6 Proteome analysis

Leaves of *sinrx1* mutants (M-3) and Jingu 21 (WT; Jin21) plants, which were normally watered and drought-treated were collected. The sampling method was based on transcriptome sample sampling, including three biological replicates, and proteomic analysis was conducted. Sample extraction, protein identification, and quantification were conducted by Novogene Co., Ltd (Beijing, China). The Vanquish™ Neo UHPLC -Astral liquid DIA method was employed to analyze the sample extract. Based on the foxtail millet protein database (https://phytozome-next.jgi.doe.gov/info/Sitalica_v2_2), the raw files were searched and analyzed using the DIA-NN software. The quantitative protein results were statistically analyzed through t-test. When Fold Change > 2.0 & $p < 0.05$, up-regulated expression proteins were screened out, and when Fold Change < 0.50 & $p < 0.05$, down-regulated expression proteins were screened out. All the differentially expressed protein (DEPs) were mapped to the Gene Ontology (GO) database (<http://www.geneontology.org/>), and for each term, the number of proteins was computed. Then, a hypergeometric test was employed, comparing with the background of all proteins to identify the GO terms significantly enriched with DEPs. KEGG enrichment analysis was carried out with the KEGG pathway as the unit, and the hypergeometric test was employed to identify the pathways that were significantly enriched in the differential proteins in comparison with all the identified protein backgrounds. The Interproscan (IPR) software was used to obtain the domain annotation information of the DEPs based on the Pfam database, and the Cell-mPLOC 2.0 website (<http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLOC-2/>) was employed for the annotation of subcellular localization information of DEPs.

2.7 Data statistics and analysis

Excel 2019 software (Microsoft Corporation, USA) was used for data analysis, and SPSS statistical software (v27.0, IBM China Co., LTD., Beijing, China) was used for statistical analysis. Duncan's method

of One-Way ANOVA was used to test for significant difference ($p < 0.05$ was indicated by letter marking method).

3. Result

3.1 *sinrx1* mutant plants were generated using CRISPR/Cas9 system

To verify the drought resistance of *SiNRX1* in foxtail millet, the CRISPR/Cas9 system was employed to knockout the *SiNRX1*. A total of 24 independent plants were obtained in the T_0 generation, and insertion/deletion mutations were identified in 4 individual plants, encompassing four types of editing. These included an eight-base deletion, a one-base insertion, a ten-base deletion, and a one-base deletion (Fig. 1A). The positive strains were replicated up to the T_4 generation, and four pure lines with distinct editing types were obtained in the greenhouse in Chifeng, China. Deletions of eight bases, insertion of one base, deletion of ten bases, and deletion of one base were respectively named *mutant-1* (M-1), *mutant-2* (M-2), *mutant-3* (M-3) and *mutant-4* (M-4). The expression of the *SiNRX1* was detected by RT-qPCR and the results indicated that M-1, M-2, M-3 and M-4 were 0.32, 0.22, 0.25 and 0.34 times that of wild type (WT) plants, respectively (Fig. 1B). The knockout of *SiNRX1* using the CRISPR/Cas9 system led to a reduction in the transcriptional expression of *SiNRX1*. Furthermore, M-1, M-2, and M-3 were selected for further study.

3.2 Response of the *sinrx1* mutant and the WT to drought stress at the germination and seedling stages

To assess the drought resistance of *sinrx1* mutant lines, the experiments were performed at germination and seedling stages. In the control group, the germination rates of the *sinrx1* mutant lines M-2 (81.3%) and M-3 (78.7%) were markedly lower than that of WT (95.3%), while no significant difference in the germination rate was observed between M-1 (95.3%) and WT (95.3%). After 7 days of treatment with 20% PEG 6000, the germination rate of *sinrx1* mutant lines M-1 (68.7%), M-2 (45.3%) and M-3 (60.7%) was significantly lower than that of WT (92.7%) plants (Fig. 1C). In the control group, the root lengths of M-2 (3.99 cm) and M-3 (4.08 cm) were significantly shorter than that of WT (5.01 cm), but no significant difference in root length was found between M-1 (4.71 cm) and WT (5.01 cm). After 7 days of treatment with 20% PEG 6000, the lengths of roots of M-1 (2.65 cm), M-2 (1.97 cm) and M-3 (1.99 cm) were significantly decreased compared with the WT (3.54 cm) plants (Fig. 1D). In the control group, the shoot lengths of M-2 (6.01 cm) and M-3 (5.96 cm) were significantly shorter than that of WT (7.96 cm), while no significant difference in shoot length was detected between M-1 (7.26 cm) and WT (7.96 cm). After 7 days of treatment with 20% PEG 6000, the lengths of shoots of M-1 (5.70 cm), M-2 (4.62 cm) and M-3 (5.20 cm) were also significantly decreased compared with the WT (6.95 cm) plants (Fig. 1E). After the 20% PEG 6000 drought simulation treatment, the germination rate, root length and shoot length of the *sinrx1* mutant lines M-1, M-2 and M-3 were significantly lower than those of WT (Fig. 1C-1E), suggesting

that the drought resistance of the *sinrx1* mutants during the germination period was inferior to that of WT.

The *sinrx1* mutant wilted to a greater extent than the WT after 12 days of drought stress treatment, and the WT recovered more effectively than the *sinrx1* mutant after 3 days of rewatering (Fig. 2A). Under drought stress, the survival rates of the *sinrx1* mutant lines M-1 (88.9%), M-2 (73.6%) and M-3 (86.1%) were significantly lower than that of WT (95.8%) plants (Fig. 2B). Under normal treatment, the soil and plant analyzer development (SPAD) values of M-1 (15.24), M-2 (14.88), and M-3 (15.16) were significantly lower than that of WT (17.3) plants (Fig. 2C). Under drought stress, The SPAD values of M-1 (14.98), M-2 (14.27) and M-3 (14.81) were significantly lower than that of WT (16.50). This indicates that *SiNRX1* might mediate the chlorophyll-related pathways in plants, resulting in a significantly lower chlorophyll content in *sinrx1* mutant plants under normal conditions compared to WT plants. Under normal conditions, there were no significant differences in proline content, POD activity, CAT activity and MDA content among the *sinrx1* mutant lines. Under drought stress, the proline contents of M-1 (166.53 µg/g), M-2 (179.44 µg/g), and M-3 (162.66 µg/g) were significantly lower than that of the WT (210.39 µg/g); the POD activities of M-1 (204.31 U/g), M-2 (183.75 U/g) and M-3 (218.53 U/g) were significantly lower than that of the WT (298.53 U/g); the CAT activities of M-1 (565.25 U/g), M-2 (417.12 U/g), and M-3 (548.29 U/g) were significantly lower than that of the WT (635.31 U/g); the MDA contents of M-1 (40.36 nmol/g), M-2 (37.20 nmol/g), and M-3 (45.61 nmol/g) were significantly higher than that of the WT (27.88 nmol/g) (Fig. 2-6D-G). These results suggest that the drought resistance of *sinrx1* mutant plants at the seedling stage is lower than that of the WT.

3.3 Analysis of the regulation of drought resistance mediated by *SiNRX1* through RNA-seq

To analyze the responses of *sinrx1* mutants and WT (Jin21) to drought, we carried out transcriptome sequencing on the leaves of WT and *sinrx1* mutants (M-3) under control treatment (CK) and drought stress treatment (DS), namely Jin21_CK, M_CK, Jin21_DS and M_DS respectively, and performed three biological replicates, with a total of 12 samples. A total of 39221040 to 50232842 original reads were generated through RNA sequencing, and 38401500 to 48978330 clean reads were obtained by filtering the original data (Table S2). The mapping rate of each sample exceeded 90% when compared with the foxtail millet genome database, and this was utilized for the subsequent analysis (Table S2). A total of 1134 novel genes were assembled by StringTie software, and these novel genes were annotated through Pfam, SUPERFAMILY, GO, KEGG and other databases. The annotation revealed that some of the novel genes belonged to the WAKY, GARS, ERF and C3H families. Some of the novel genes were mainly enriched in the metabolic process (GO:0008152) during BP and in metabolic pathways (sita01100) in KEGG (Table S3).

Quantitative analysis of gene expression levels was carried out for all samples, and correlation coefficients of intra-group and inter-group samples were computed based on the FPKM values of all genes in each sample. The intra-group R^2 was higher than 0.95, suggesting that the intra-group

expression patterns were close (Fig. 3A). In principal component analysis (PCA), samples between groups were dispersed, while samples within groups were clustered together, indicating that the biological replication within groups was satisfactory (Fig. 3B). By adopting $|\log_2(\text{FoldChange})| \geq 1$ & $\text{padj} \leq 0.05$ as the screening criteria, 3910 differentially expressed genes (DEGs) were identified in Jin21_DSvsJin21_CK and 5264 DEGs in M_DSvsM_CK. Additionally, 596 DEGs were found in M_CKvsJin21_CK (Fig. 3C). The sum of all the numbers within the circle in Venn's diagram represents the total quantity of differential genes in the comparison combination, and the overlapping area indicates the number of common differential genes between the combinations. It is revealed that the three comparison groups have 85 common differential genes, including 8 ERF transcription factors, 5 C2H2 members, and 4 heat shock transcription factors (HSF) (Fig. 3D; Table S4). The distribution and difference of DEGs are presented through the volcano map (Fig. S1; Table S4).

The Venn diagram indicates that 2253 DEGs (1179 up-regulated DEGs and 1074 down-regulated DEGs) are exclusive to the M_DSvsM_CK group (Fig. 3D; Table S4). This suggests that the 2253 DEGs are drought-responsive genes specifically influenced by *SiNRX1*. Additionally, gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed on these 2253 DEGs. The GO enrichment analysis of 2253 DEGs reveals that, within the biological process (BP) category, these genes are primarily enriched in regulation of hormone levels (GO:0010817), hormone metabolic process (GO:0042445), amine metabolic process (GO:0009308), cellular carbohydrate biosynthetic process (GO:0034637), carbohydrate biosynthetic process (GO:0016051), aromatic amino acid family metabolic process (GO:0009072), cytokinin metabolic process (GO:0009690), cellular hormone metabolic process (GO:0034754) and disaccharide biosynthetic process (GO:0046351) ($p < 0.01$) (Fig. 4; Table S5). In the cell components (CC) category, 2253 DEGs are predominantly enriched in protein phosphatase type 2A complex (GO:0000159), protein serine/threonine phosphatase complex (GO:0008287) and phosphatase complex (GO:1903293) ($p < 0.05$) (Fig. 4; Table S5). In the molecular function (MF) category, 2253 DEGs are predominantly enriched in DNA-binding transcription factor activity (GO:0003700), cytokinin dehydrogenase activity (GO:0019139), cellulose synthase activity (GO:0016759; GO:0016760), protein phosphatase regulator activity (GO:0019888; GO:0019208), carbohydrate binding (GO:0030246), oxidoreductase activity (GO:0016645), transferase activity (GO:0008194; GO:0016747; GO:0015020; GO:0035251; GO:0015018; GO:0008374) and hydrolase activity (GO:0016811) ($p < 0.05$) (Fig. 4; Table S5).

The KEGG pathways of the 2253 DEGs were significantly enriched in phenylalanine, tyrosine and tryptophan biosynthesis (sita00400), plant hormone signal transduction (sita04075), phenylpropanoid biosynthesis (sita00940), plant-pathogen interaction (sita04626), starch and sucrose metabolism (sita00500), pyruvate metabolism (sita00620), biosynthesis of amino acids (sita01230), zeatin biosynthesis (sita00908) and diterpenoid biosynthesis (sita00904) ($p < 0.05$) (Fig. 5; Table S6).

3.4 RT-qPCR was employed to validate the reliability of RNA-seq

To validate the reliability of the transcriptome, four up-regulated genes and four down-regulated genes that were consistently expressed in all 12 samples were selected for transcriptome verification. The results indicated that the RT-qPCR and transcriptome data were in agreement (Fig. 6A-6C), thereby confirming the reliability of the transcriptome analysis.

3.5 Analysis of the regulation of drought resistance mediated by *SiNRX1* through DIA quantitative proteomic

To reveal the response of *SiNRX1* to drought stress at the protein level, DIA quantitative proteomics was carried out on the leaves of WT (Jin21) and *sinrx1* mutant (M-3) under normal treatment and drought treatment. A total of 104424 peptides and 11122 proteins were identified by DIA, and 10219 to 10905 proteins were identified in 12 samples (Fig. 7A). The Coefficient of Variance (CV) was employed to measure the degree of variation of the observed values in the samples. The smaller the CV value, the better the repeatability, and the results suggest good repeatability (Fig. 7B).

The proteins co-identified in the two groups of samples were analyzed, among which Jin21_DSvsJin21_CK, M_DSvsM_CK and M_CKvsJin21_CK co-identified 10343, 10347 and 10180 proteins, respectively. When $FC > 2.0$ & $P\text{-value} < 0.05$, up-regulated expression proteins were screened, and when $FC < 0.5$ & $P\text{-value} < 0.05$, down-Regulated expression proteins were screened. The results indicated that 239 differential expression proteins (DEPs) were identified in the Jin21_DSvsJin21_CK, including 233 up-regulated proteins and 6 down-regulated proteins (Fig. 7C, Table S7). Meanwhile, 221 DEPs were identified in the M_DSvsM_CK, including 188 up-regulated proteins and 33 down-regulated proteins (Fig. 7C, Table S7). A total of 159 DEPs were identified in the M_CKvsJin21_CK, including 105 up-regulated proteins and 54 down-regulated proteins (Fig. 7C, Table S7). The distribution and differences of DEPs among the three groups were presented in the volcano map (Fig. S2). Venn diagram results indicated that the three groups had 8 common DEPs (Fig. 7D). They were Seita.3G209800, Seita.3G228400, Seita.3G268600, Seita.5G182500, Seita.5G208900, Seita.5G263500, Seita.9G034000 and Seita.9G567800 (Table S7). The GO annotations of Seita.5G182500, Seita.5G263500 and Seita.9G567800 are respectively redox activity (GO:0016491), protein disulfide redox activity (GO:0015035) and serine endopeptidase activity (GO:0004252) in MF, and the KOGs annotations include lipid transport and metabolism, post-translational modification, protein conversion, molecular chaperone and signal transduction mechanisms respectively.

The Venn diagram indicates that 155 DEPs (127 up-regulated DEPs and 28 down-regulated DEPs) are exclusive to the M_DSvsM_CK group (Fig. 7D; Table S7). This suggests that the 155 DEPs are drought-responsive proteins specifically influenced by *SiNRX1*. To analyze the main biological functions of 155 DEPs, GO enrichment was carried out on DEPs, and the significantly enriched GO term in DEPs was plotted with $p < 0.05$ as the threshold (Fig. 8; Table S8). The 155 DEPs were enriched in a total of 29 biological process (BP), 8 cellular component (CC), and 50 molecular function (MF) categories (Table S8). They were significantly enriched in cell wall organization (GO:0071555), oxidation-reduction process (GO:0055114), chlorophyll catabolic process (GO:0015996), proteasome-mediated ubiquitin-dependent

protein catabolic process (GO:0043161) and asparagine biosynthetic process (GO:0006529) within BP ($p < 0.05$) (Fig. 8; Table S8). The 155 DEPs were significantly enriched in the cell wall (GO:0005618) within the CC ($p < 0.05$) (Fig. 8; Table S8). In addition, they were significantly enriched in carboxylic ester hydrolase activity (GO:0052689), heme binding (GO:0020037), iron ion binding (GO:0005506), enzyme regulator activity (GO:0030234) and protein dimerization activity (GO:0046983) within the MF ($p < 0.01$) (Fig. 8; Table S8).

To identify the main biochemical metabolic pathways and signal transduction pathways in which the 155 DEPs were involved, KEGG enrichment analysis of 155 DEPs was carried out, and the top 20 significantly enriched KEGG pathways were selected for bubble plots (Fig. 9). The DEPs were enriched in 37 KEGG pathways. Among them, the biosynthesis of secondary metabolites (map01110), brassinosteroid biosynthesis (map00905), metabolic pathways (map01100), ascorbate and aldarate metabolism (map00053), phenylpropanoid biosynthesis (map00940), indole alkaloid biosynthesis (map00901), flavonoid biosynthesis (map00941), ether lipid metabolism (map00565), flavone and flavonol biosynthesis (map00944) and glycerophospholipid metabolism (map00564) were significantly enriched ($p < 0.05$) (Fig. 9; Table S9). The pathways of biosynthesis of secondary metabolites (map01110) and metabolic pathways (map01100) had the most DEPs enriched, with 19 and 25 DEPs respectively (Fig. 9; Table S9).

Protein domain enrichment can identify the protein domains that are statistically significantly enriched. Moreover, differences in protein domains might be accountable for the variations. The results demonstrated that the 155 DEPs were significantly enriched in cytochrome P450 (IPR001128), CCCH-type zinc finger (IPR000571), putative polysaccharide biosynthesis protein (IPR021148), O-methyltransferase (IPR001077), multicopper oxidase (type 1, IPR001117; type 2, IPR011706; type 3, IPR011707) and phospholipid/glycerol acyltransferase (IPR002123) ($p < 0.01$) (Fig. S3; Table S10). To understand the functional regions of the 155 DEPs, the subcellular localization of the 155 DEPs was predicted. Moreover, a total of 63 DEPs had their subcellular localization information, and they were located in the cell membrane (12), nucleus (12), cytoplasm (10), cell wall (5), endoplasmic reticulum protein (4), chloroplast (4), extracell (4), golgi apparatus (4), mitochondrion (4), vacuole (3) and peroxisome (1) (Fig. S4; Table S11).

3.6 Analysis of the regulation of drought resistance mediated by SiNRX1 through the combination of RNA-seq and proteome

The combined analysis of the transcriptome and proteome can disclose the post-transcriptional regulation of gene expression. The DEGs information obtained from the transcriptome was integrated with the DEPs information identified by the proteome, and the corresponding relationship was determined and depicted as the Venn diagram (Fig. 10). The results indicated that 14 pairs of DEGs and DEPs had transcriptional levels corresponding to protein level expression. Among them, 12 pairs had transcriptional levels consistent with protein level expression, including 10 pairs of both DEGs and DEPs with up-regulated expression. Seita.5G195800 (PP2C phosphatase) and Seita.J003100 (peroxidase) had

down-regulated expression of DEGs and DEPs, while Seita.4G283200 and Seita.5G386600 had down-regulated expression at the transcriptional level and up-regulated expression at the protein level (Fig. 10; Table S12). The GO functional annotations of these genes/proteins are as follows: oxidation-reduction process, catalytic activity, metabolic process, carboxylic ester hydrolase activity, sequence-specific DNA binding and single-organism process. In terms of KEGG, the enrichment mainly occurs in biosynthesis of secondary metabolites (map01110) and metabolic pathways (map01100) (Table S12).

4. Discussion

NRX, a member of the TRX subfamily, has the electron transfer function and participates in plant growth and development, as well as responses to biotic and abiotic stresses [25, 27, 28, 30–32]. *SiNRX1*, *SiNRX2* and *SiNRX3* were identified in the genome of foxtail millet, among which *SiNRX1* possesses a typical TRX domain (with WCPPC and WCGPC redox active sites). The overexpression of *SiNRX1* in *Arabidopsis* enhanced the drought resistance of transgenic *Arabidopsis thaliana* [3]. However, it remains unclear whether *SiNRX1* is implicated in regulating the drought resistance of foxtail millet. In this study, the CRISPR/Cas9 system was utilized to knock out *SiNRX1* to generate *sinrx1* mutant. After a 7 day treatment with 20% PEG 6000 during the germination period, the germination rate, root length, and shoot length of *sinrx1* mutant lines M-1, M-2, and M-3 significantly decreased compared with the WT, indicating that the drought resistance of *sinrx1* mutant strains during the germination period was lower than that of the WT. After 12 days of drought stress treatment during the seedling stage, the wilting degree of the *sinrx1* mutant was significantly more severe compared to that of the WT. Furthermore, three days after rehydration, the recovery degree of the WT was superior to that of the *sinrx1* mutant. After drought stress, the survival rate, chlorophyll content, proline content, POD activity, and CAT activity of the *sinrx1* mutant were significantly lower than those of the WT. Meanwhile, the MDA content was significantly higher than that of the WT. This suggests that the drought resistance of the *sinrx1* mutant during the seedling stage is lower than that of the WT. *SiNRX1* might regulate drought resistance by participating in the antioxidant system to eliminate ROS. The overexpression of *SiNRX1* in *Arabidopsis thaliana* enhanced the drought resistance of the transgenic plants, while knocking out *SiNRX1* in foxtail millet decreased the drought resistance during the germination and seedling stages. *SiNRX1* positively regulates drought resistance.

Seed development and germination are accompanied by extensive alterations in the redox state of seed storage proteins [50–54]. Studies have demonstrated that the NTR/TRX h system and the mitochondrial TRX system exert significant functions in the germination of certain plant seeds, and the redox alterations mediated by TRX are implicated in regulating the germination and emergence of seeds [53–56]. In this study, the germination rates, root lengths, and shoot lengths of the *sinrx1* mutant M-2 and M-3 lines in the control group were significantly lower than those of WT (Fig. 1). Therefore, *SiNRX1* might play a certain role in regulating the seed germination process. However, the performance variations among different *sinrx1* mutant strains might be ascribed to the different editing types of *SiNRX1*. *SiNRX1* might regulate seed germination by adjusting the redox state of seed reserve proteins, but the specific mechanism still requires further exploration.

TRX is a redox enzyme that participates in key processes related to photosynthetic activity, redox homeostasis, and antioxidant mechanisms within chloroplasts of plants [14, 57]. NRX is a member of the TRX superfamily with redox sites, and NRX may have a similar function to TRX [15, 25]. In this study, the chlorophyll content of the *sinrx1* mutant at the seedling stage under normal treatment and drought treatment was significantly lower than that of WT plants. Proteomic analysis indicated that, based on GO enrichment analysis, 155 DEPs were significantly enriched in the chlorophyll catabolic process. Among these, Seita.2G134400, a gene related to chlorophyll catabolic process, was identified as one of the 10 upregulated gene-protein pairs (DEGs and DEPs). This suggests that the upregulation of chlorophyll-degrading enzymes may result in a decline in chlorophyll content and a reduction in drought resistance in the *sinrx1* mutant under drought treatment. Nevertheless, additional research is still required to elucidate the specific mechanism underlying the reduction in chlorophyll content in the *sinrx1* mutant leaves under normal or drought treatment.

Phenylpropanoids are a group of chemicals originated from phenylalanine, constituting a structurally diverse set of secondary metabolites (such as flavonoids and lignin) which play a crucial role in responding to biological and abiotic stresses [58–60]. The initial steps of the phenylpropanoid pathway are jointly referred to as the general phenylpropanoid pathway (GPP) [59, 61, 62]. The first enzymatic step involves the deamination of phenylalanine by phenylalanine ammonia-lyase (PAL), yielding cinnamic acid. Subsequently, various secondary metabolite products are generated through other reactions [63–67]. Furthermore, secondary metabolites mainly eliminate ROS to safeguard plants from lipid peroxidation, synthesize and transport metabolites and enzymes, stabilize cell components, convey signals and regulate related gene expression, and possess other defense functions [68, 69]. In this research, DEGs and DEPs were significantly and jointly enriched in the phenylpropanoid biosynthesis pathway. Significantly, both the Seita.7G168800 and Seita.J003100 genes/proteins were detected in the phenylpropanoid metabolic pathway that was jointly enriched by DEGs and DEPs. In addition, Seita.7G168800, exhibits a protein sequence similarity of 77% with that of *Arabidopsis* AT3G53260 (AtPAL2). Studies have indicated that AtPAL2 possesses the catalytic ability for L-phenylalanine deamination [70, 71], and Seita.7G168800 may have similar functions to AtPAL2. The Seita.J003100 gene, which encodes a secondary metabolite (Peroxidase), shows downregulation at both the transcriptional and protein levels. Significantly, peroxidase (POD) activity, which is crucial for alleviating oxidative stress, was markedly reduced in the *sinrx1* mutant compared with WT plants under drought conditions. This is in line with the alterations in the expression of peroxidase genes/proteins before and after drought stress, as indicated by the RNA-seq/proteomic results. These findings indicate that SiNRX1 might mediate the phenylpropanoid biosynthesis pathway and contribute to the regulation of drought resistance in foxtail millet.

TRX regulates the structure and biological functions of related proteins by catalyzing the formation and reduction of disulfide bonds in specific target proteins in different cellular compartments [72–74]. In recent years, with the rapid development of proteomics technology and the continuous improvement of protein labeling techniques and affinity chromatography, certain progress has been made in the identification of TRX target proteins [75]. Yoshida et al. (2013) detected 101 potential TRX target proteins

in the mitochondria of *Arabidopsis*, spinach, and potato by using affinity chromatography in combination with proteomics techniques. These proteins are implicated in the respiratory chain, TCA cycle, photorespiration, amino acid metabolism, redox regulation system, antioxidant defense, etc. They include dehydrogenases, transferases, oxidoreductases (such as TRX), protein kinases, etc [76]. As a member of the TRX superfamily, NRX features a conserved CXXC redox-active site. This structural trait suggests that it may recognize similar target proteins in a way analogous to TRX. In this study, the results of transcriptome and proteome analyses indicate that 14 pairs of DEGs and DEPs had transcriptional levels corresponding to protein level expression. These 14 proteins included protein phosphatases, dehydrogenases, transferases, etc. It is highly probable that these 14 proteins contain the target proteins of SiNRX1. Among them, Seita.5G195800 (PP2C phosphatase) and Seita.J003100 (peroxidase) had down-regulated expression of DEGs and DEPs. Moreover, Seita.5G346500 (SiNRX2) is upregulated at both the transcriptional and protein levels. Research has demonstrated that the subcellular localization information of SiNRX2 is consistent with that of SiNRX1 [3]. In addition, *SiNRX2* shows certain responses under simulated drought stress, suggesting that SiNRX2 and SiNRX1 might act synergistically in the drought response of foxtail millet. Moreover, Seita.6G105300 (UDP-glucose 6-dehydrogenase), a constituent of the oxidative pentose phosphate pathway, is upregulated at both the transcriptional and protein levels. Research has indicated that TRX is related to the activity of glucose 6-phosphate dehydrogenase [75, 77]. The findings suggest that Seita.5G195800 (PP2C phosphatase), Seita.J003100 (peroxidase), Seita.5G346500 (SiNRX2), Seita.6G105300 (UDP-glucose 6-dehydrogenase) could be a potential target protein of NRX. However, additional experiments such as co-immunoprecipitation (co-IP) are required for verification.

5. Conclusion

Knocking out *SiNRX1* using CRISPR/Cas9 system decreased the drought resistance of foxtail millet during the germination and seedling stages. The findings from transcriptomics and proteomics suggest that the expression of related genes/proteins under drought stress is specifically influenced by SiNRX1. Moreover, these DEGs/DEPs are significantly and collectively enriched in the phenylpropanoid biosynthesis pathway. This study is beneficial for comprehending the pathways by which NRX takes part in regulating the drought resistance of foxtail millet, and offers theoretical guidance for the breeding of drought-resistant foxtail millet varieties.

Declarations

Supplementary Materials: The following supporting information can be downloaded at:

Author Contributions: Conceptualization, X.Z. and J.X., methodology, X.C., S.Z., J.Z. and T.S., soft-ware, X.C., S.Z., J.R., X.W., Y.B.; investigation, X.C., S.Z., J.R., X.W., Y.B. and X.L.; visualization, X.C., J.Z. and T.S.; validation, X.C., S.Z., J.Z. and T.S.; resources, J.X.; data curation, X.C., Y.W., X.L. and C.C.; writing original draft preparation, X.C.; writing review and editing, X.Z. and J.X.. All authors have read and agreed to the published version of the manuscript.

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Figures

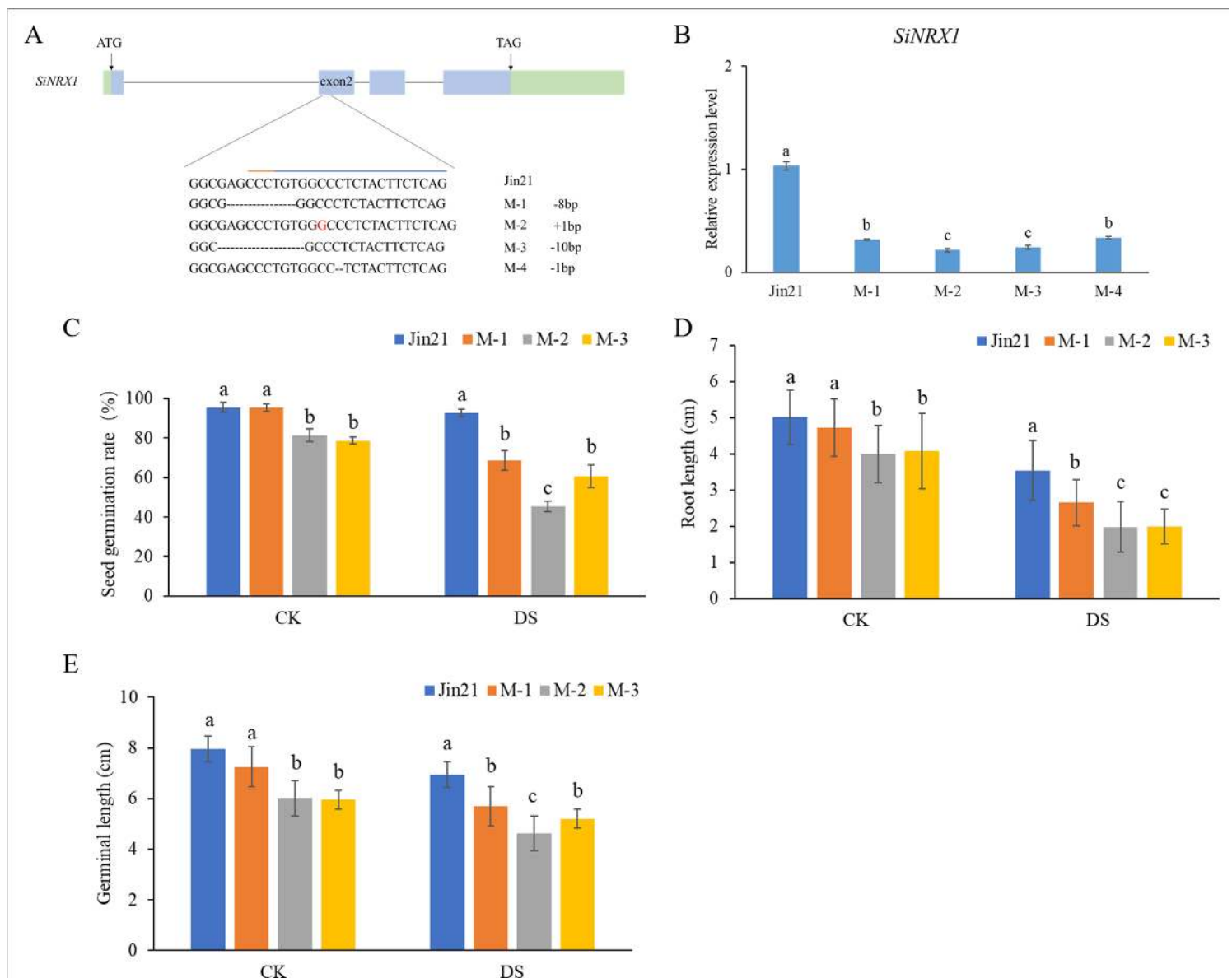


Figure 1

Knockout of *SiNRX1* decreased the drought resistance of foxtail millet at the germination stage. **(A)** The target region of the gRNA and the editing type for the *SiNRX1* genome. Exons are represented by blue boxes, while introns are depicted as lines. The ATG and TGA sequences indicate the start and stop codons, respectively. The orange segment denotes the PAM sequence, the blue segment corresponds to the sgRNA sequence, the dashed line signifies missing nucleotide sequences, and the red bases represent insertion sequences; **(B)** The expression level of *SiNRX1* in *sinrx1* mutant, and the standard deviation of *SiNRX1* expression level is represented by error bars (mean $\pm$ SD, n=3); **(C-E)** Identification of drought resistance of *sinrx1* mutant at germination stage. **(C)** Seed germination rate; **(D)** Root length measurement taken 7 days after seed germination; **(E)** Shoot length measurements taken 7 days after seed germination. The standard deviation of seed germination rate was represented by an error bar (mean $\pm$ SD, n=3), and the standard deviation of root length and bud length was represented by an error bar (mean $\pm$ SD, n=30). Significance was determined by Duncan test of one-way ANOVA, with letters indicating $p < 0.05$.

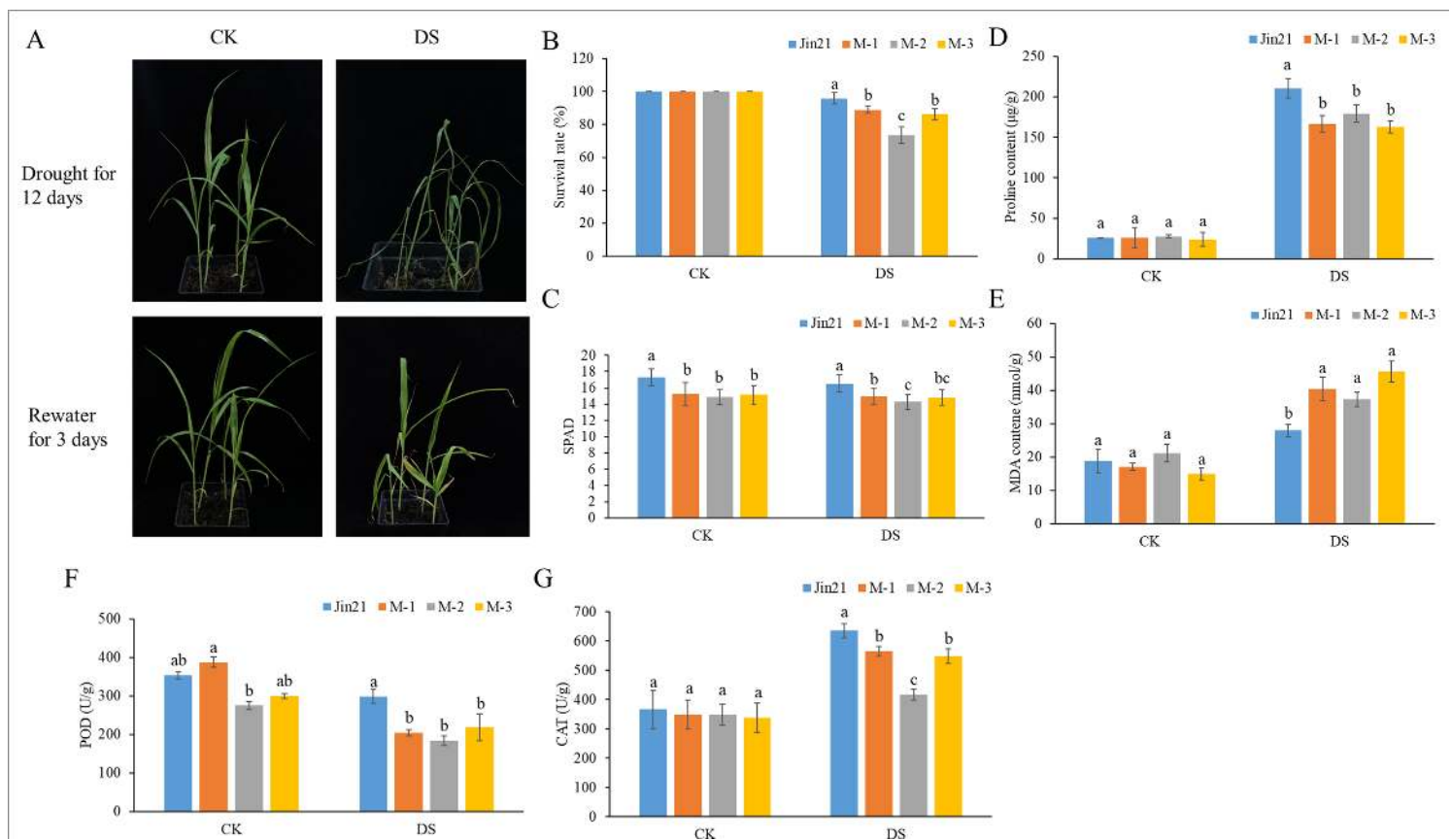


Figure 2

Identification of drought resistance of *sinrx1* mutant at seedling stage. **(A)** Drought resistance phenotype of the *sinrx1* mutant. Jin21 and the *sinrx1* mutants were planted in the same pot, with Jin21 located in the upper left corner of each pot, and M-1, M-2 and M-3 respectively placed in the upper right corner, lower left corner and lower right corner; **(B)** Survival measurement, survival values are expressed as mean, standard deviation is expressed as error bars (mean±SD, n=3); **(C)** Determination of chlorophyll at the seedling stage. The SPAD value is presented as the mean value, and the standard deviation is represented as the error bar (mean±SD, n=15); **(D-G)**: Proline **(D)**, MDA **(E)**, POD **(F)** and CAT **(G)** were measured. The measured values were presented as mean values, and the standard deviations were represented as error bars (mean±SD, n=3). CK stands for the control group and DS represents the drought stress treatment group. One-way ANOVA with Duncan test was employed for significance determination, and letters indicated $p < 0.05$.

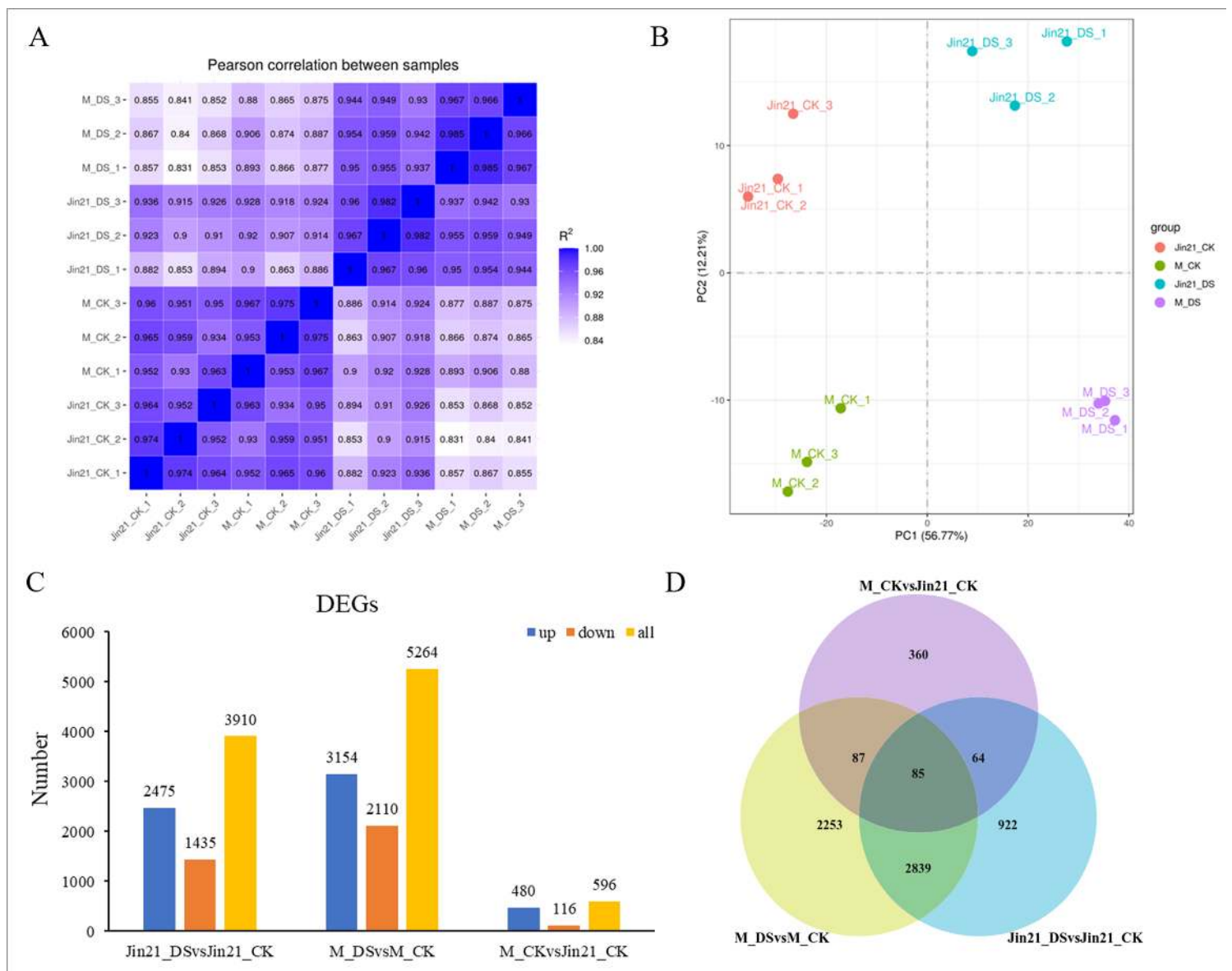


Figure 3

Correlation analysis among samples and analysis of differentially expressed genes (DEGs). **(A)** Heat map of the correlation between samples, where the horizontal and vertical coordinates represent the square of the correlation coefficients of each sample; **(B)** Plot of principal component analysis results. The abscissa represents the first principal component, and the ordinate represents the second principal component; **(C)** Statistical bar chart depicting the number of DEGs in the differential comparison combination; **(D)** Venn map of DEGs, with different colors denoting different comparative combinations.

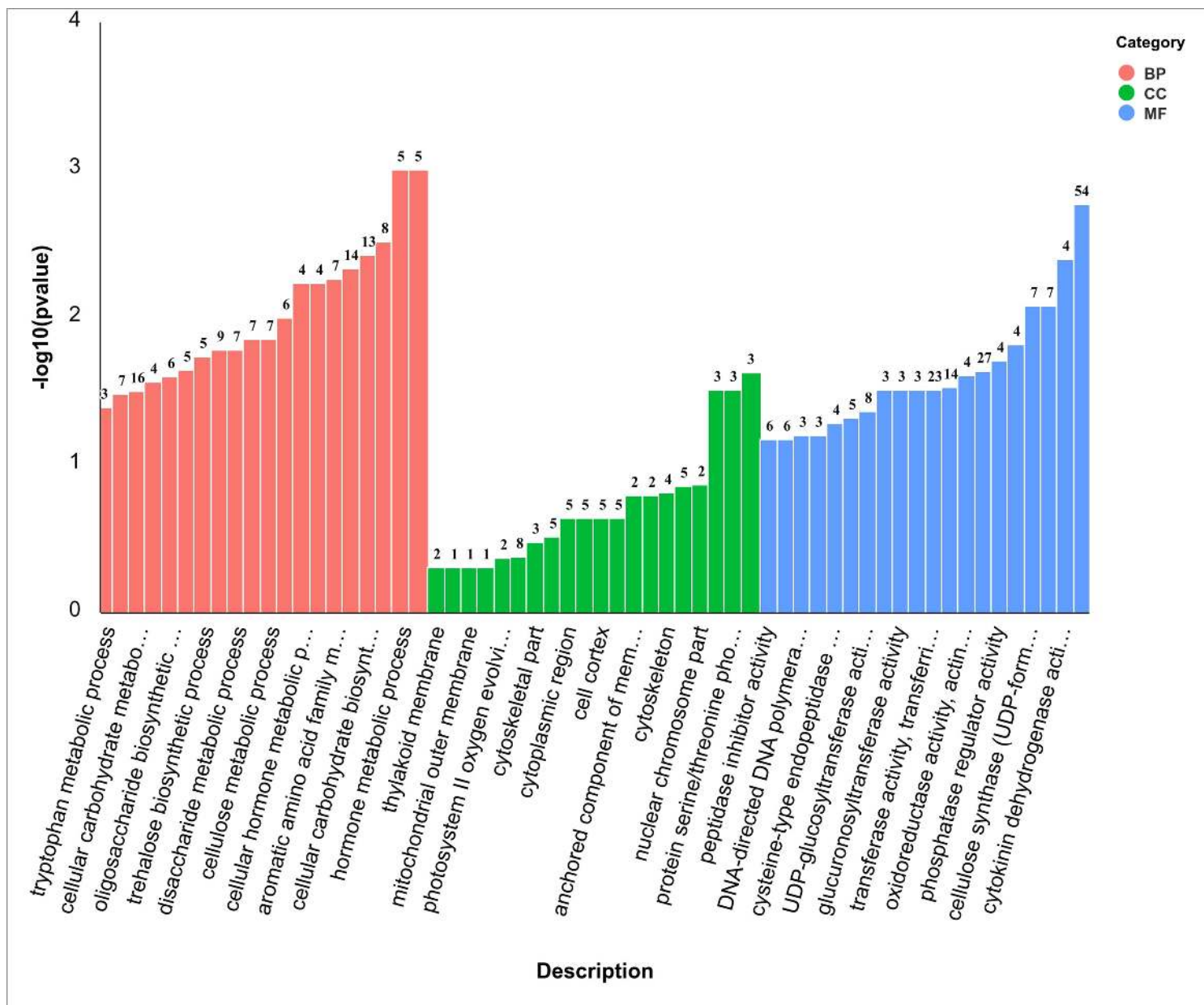


Figure 4

Histogram of the GO enrichment analysis for 2253 DEGs. The horizontal coordinate is GO Term, and the vertical coordinate represents the significance level of GO term enrichment, which is indicated by $-\log_{10}(p\text{-value})$. Different colors respectively represent different functional classifications.

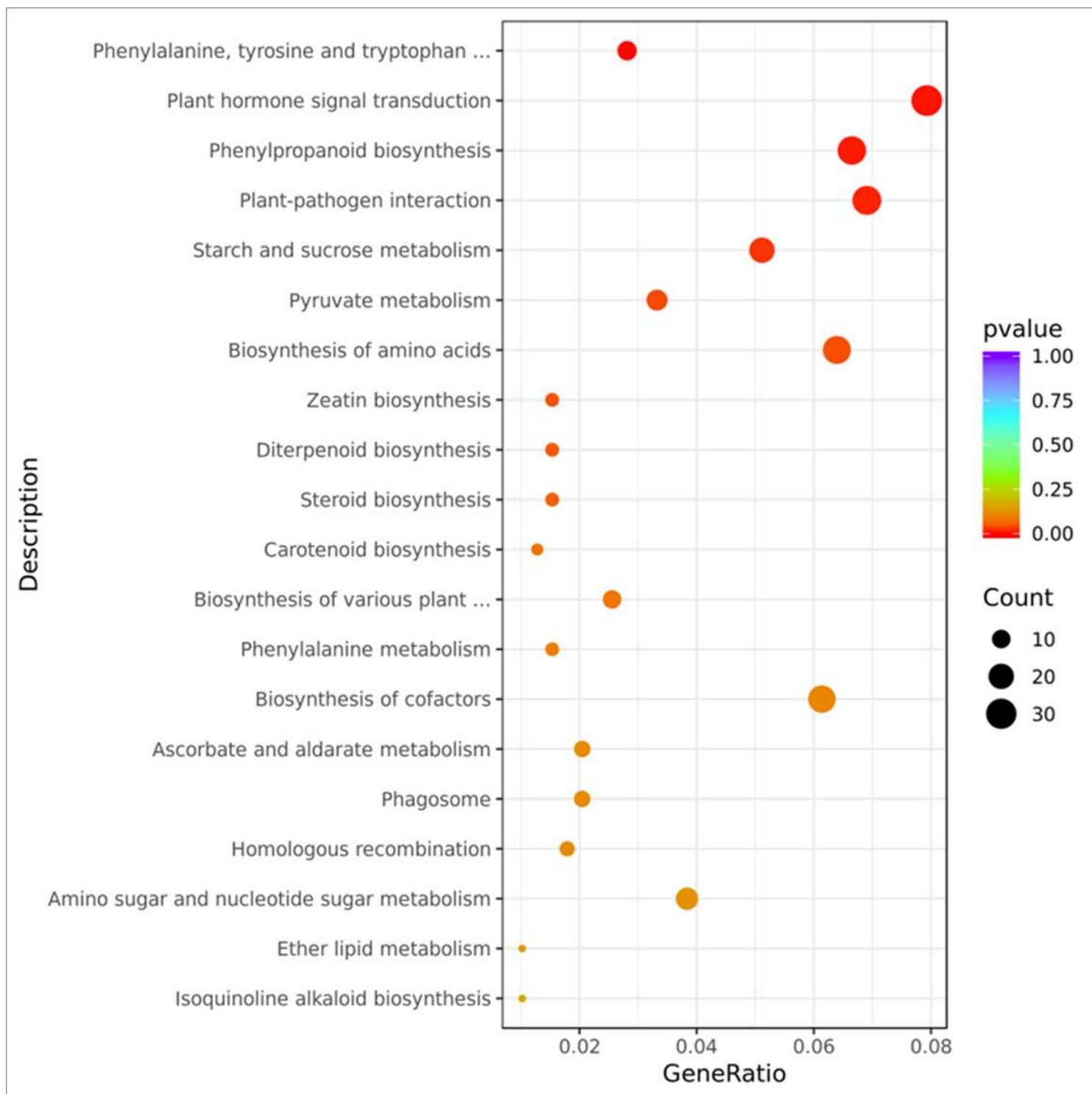


Figure 5

Scatter plot of KEGG enrichment analysis for 2253 DEGs. The abscissa represents the ratio of the number of DEGs annotated to the KEGG pathway to the total number of DEGs, and the ordinate indicates the KEGG pathway.

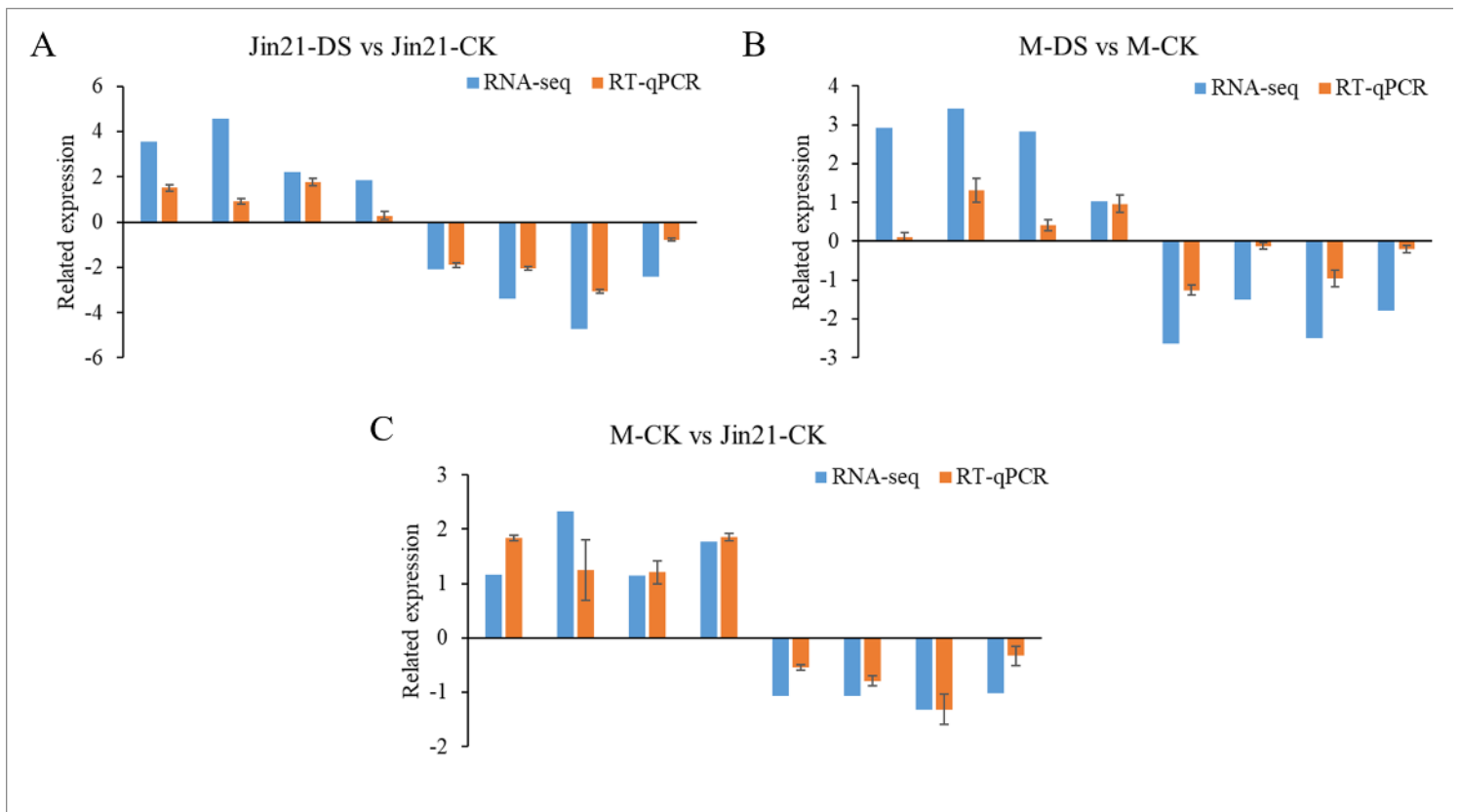


Figure 6

Eight DEGs were verified by RT-qPCR. SiActin-7 was used as the reference gene, and different genes were represented on the x axis. From left to right are Seita.2G050800, Seita_6G159400, Seita_6G187100, Seita.8G136300, Seita.2G383000, Seita_8G099000, Seita_3 G397800 and Seita_5G303900, the y axis represents the relative expression of the selected genes. The blue color denotes the $\log_2$ FPKM values derived from RNA-seq data, while the orange color indicates the $-\Delta\Delta t$ values obtained from RT-qPCR. The standard deviation is represented by error bars (mean $\pm$ SD, n = 3).

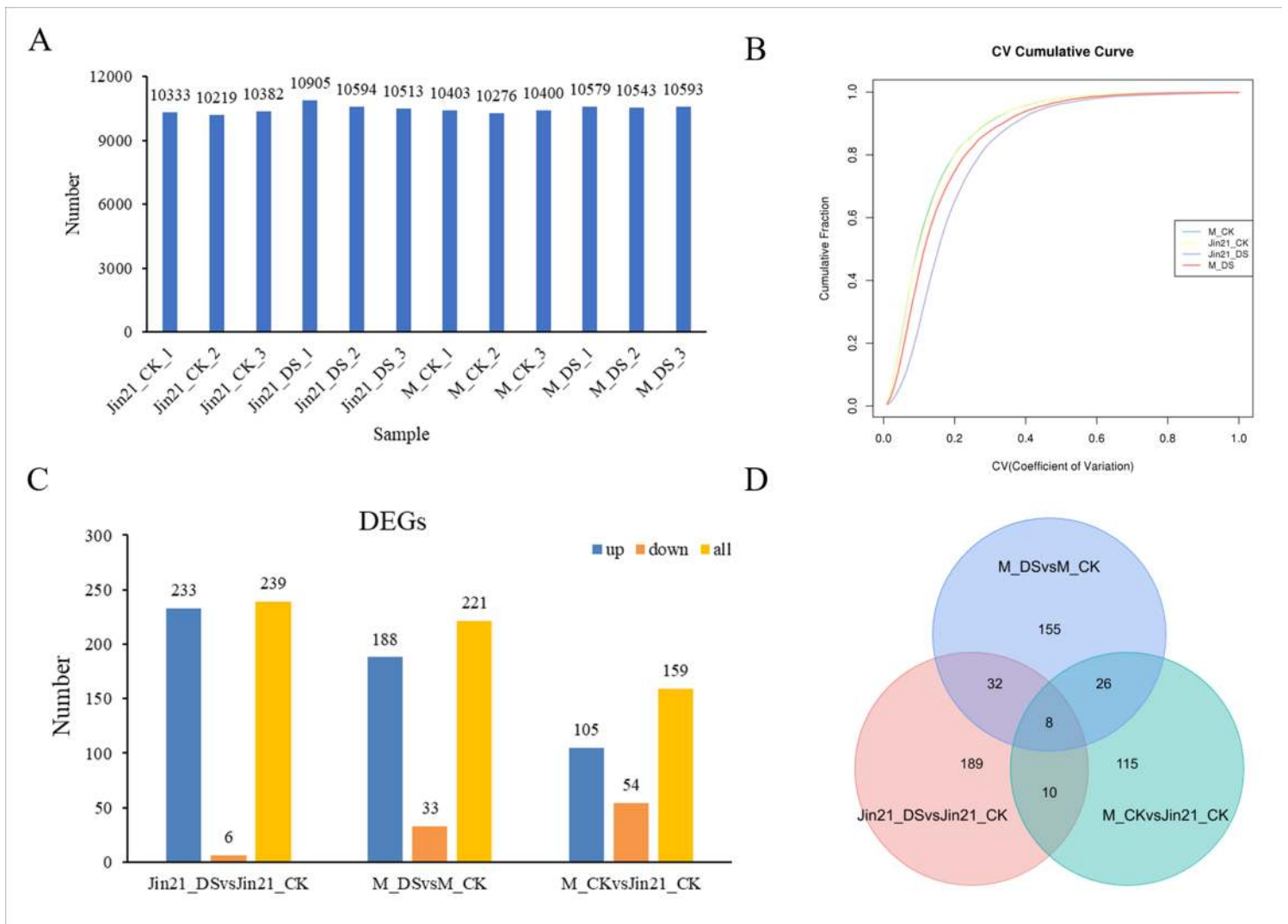


Figure 7

Sample quantitative analysis and differentially expressed protein (DEPs) analysis. **(A)** Overview of protein identification of samples. The abscissa is the sample name and the ordinate is the number of proteins; **(B)** Repeatability CV analysis; **(C)** Bar graph of DEPs analysis results; **(D)** Venn diagram of DEPs, different colors indicate different comparison combinations.

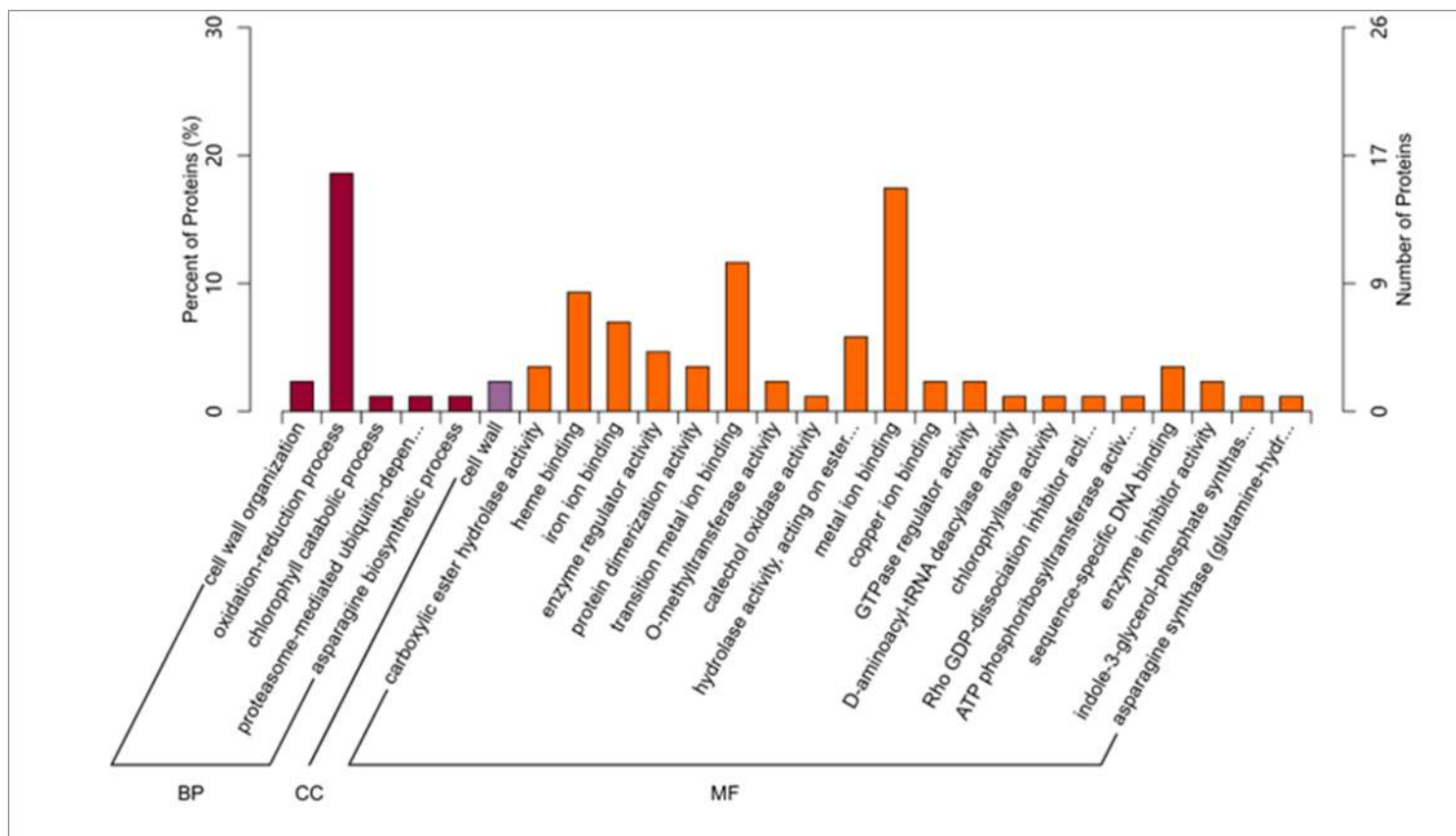


Figure 8

Histogram of the GO enrichment analysis for 155 DEPs. The figure illustrates the enrichment results across three categories, each displaying up to 20 types ($p < 0.05$). The y-axis represents the ratio of DEPs associated with a specific GO term to the total number of DEPs annotated by all GO terms.

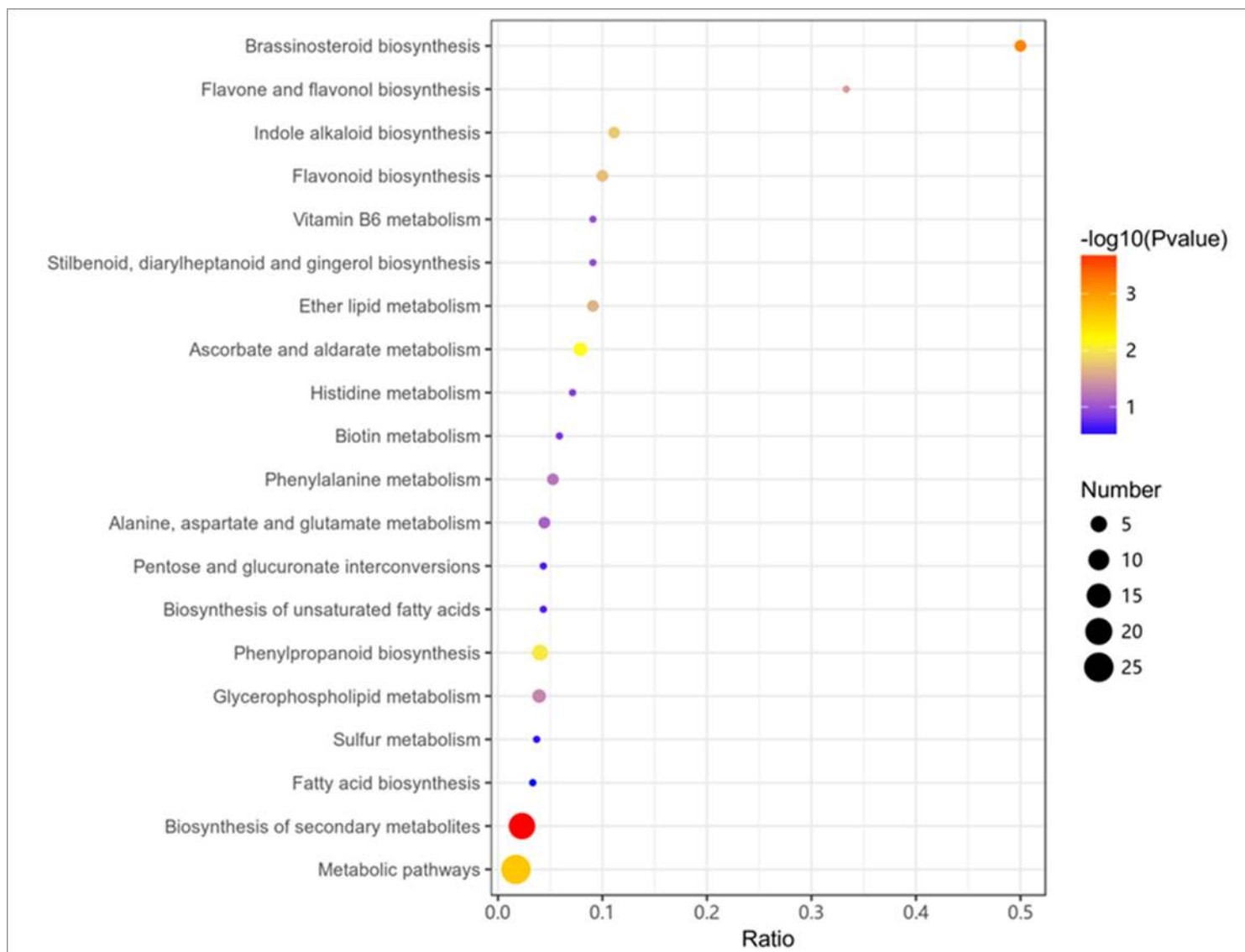


Figure 9

KEGG enrichment bubble diagram for 155 DEPs. The horizontal axis in the figure represents the ratio of DEPs within a specific pathway to the total number of proteins identified in that pathway. A higher value indicates a greater degree of enrichment for differential proteins within the pathway. The color of the dot represents the P -value of the hypergeometric test. The color ranges from blue to red, and the redder the color, the smaller the P -value, indicating the greater the reliability of the test and having statistical significance. The size of the dot represents the number of DEPs in the corresponding pathway, and the larger the dot, the greater the number of DEPs in the pathway.

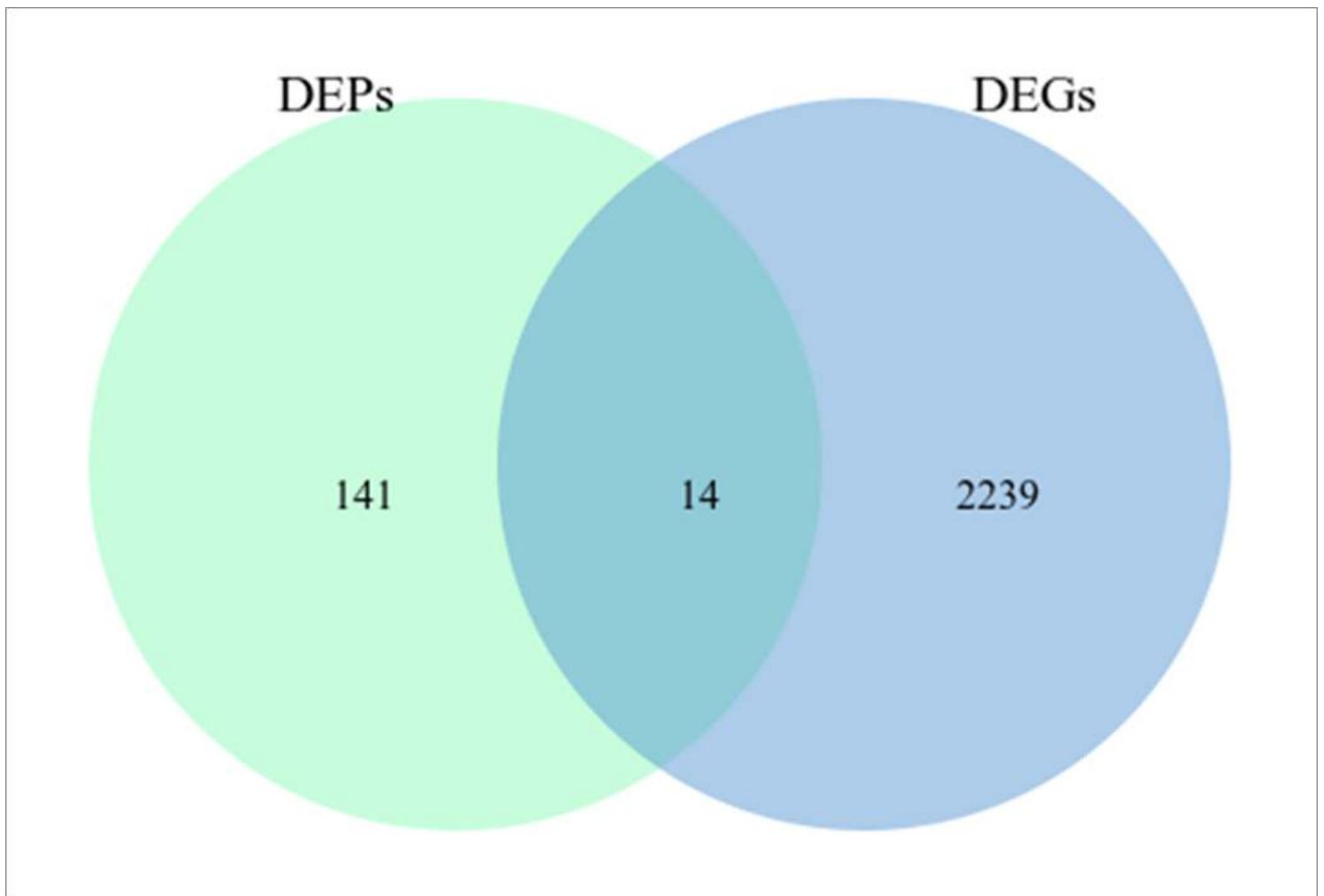


Figure 10

Venn diagram depicting the expression regulation of 2253 DEGs and 155 DEPs. The shared and specific genes/proteins are obtained according to different regions.

Supplementary Files

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- [Additionalinformation.zip](#)