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PHYSIOLOGICAL RESPONSE AND DIFFERENTIAL GENE EXPRESSION ANALYSIS OF *HEMIPTELEA DAVIDII* (ULMACEAE) SEEDLINGS UNDER PEG STRESS

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Abstract

Hemiptelea davidii is a drought-tolerant, sand-fixing, wind-preventing, and versatile plant that is highly adaptable to growing in harsh terrain such as barren hills and sandy areas. In this study, PEG-6000 solution was used to simulate drought in young *Hemiptelea davidii* seedlings to investigate the changes of chlorophyll, osmoregulation substances, malondialdehyde (MDA) and antioxidant enzyme activities in the leaves at four PEG concentrations (0, 10%, 20% and 30%). *Hemiptelea davidii* was stressed with high concentration of 30% PEG for 0h, 6h and 12h, and its leaves were collected for transcriptome sequencing to investigate the molecular mechanism of *Hemiptelea davidii* under drought stress. The experiment shows that *Hemiptelea davidii* can resist a certain extent and duration of drought. However, under high concentration and long-term drought, its defense system will be destroyed and disordered, and its drought resistance will decrease. A total of 54,598 Unigene were assembled by transcriptome sequencing analysis of *Hemiptelea davidii* leaves, and 56.4% were found to be annotated with major databases. The transcripts showed that 620 and 3803 differentially expressed genes (DEGs) were detected in w1_w2 and w1_w3, respectively. GO, COG and KEGG analyses showed that PEG stress induced the expression of genes related to photosynthesis and metabolic pathways. Transcription factor analysis revealed a total of 63 transcription factor families, including C2H2, bHLH, AP2/ERF-ERF, etc. These transcription factor genes were involved in plant hormone regulation, growth and development, and might play an essential role in *Hemiptelea davidii* coping with PEG stress. The study of the drought-tolerant mechanism of *Hemiptelea davidii* provide a basis for popularizing the planting of *Hemiptelea davidii* and excavating the stress-resistant genes of *Hemiptelea davidii*.

Key words: *Hemiptelea davidii*; Drought stress; Physiological response; Transcriptome; Differential expressed gene

Introduction

Hemiptelea davidii, a small deciduous tree, belongs to Ulmaceae family (Urticales). *Hemiptelea davidii* is a native tree species in Inner Mongolia, exists as a community-building species and forming communities in the horqin sands, which is a new sandy vegetation type in China. *Hemiptelea davidii* has the advantages of cold tolerance, barrenness, resistance to drought and low temperature, wind and sand fixation, and strong adaptability, to grow in harsh places such as barren mountains, barren slopes, and sandy areas, and also can be used as biological fences for sand prevention and control, which plays a certain role in water conservation and biodiversity protection. According to the complete chloroplast genomes, *Hemiptelea davidii* is near to Zelkova and Ulmus species in the Ulmaceae family but is a little farther away from species in the Cannabaceae, Moraceae, and Urticaceae families (Liu *et al*., 2019; Liu *et al*., 2022).

The morphological anatomy, community structure and pharmacology of the *Hemiptelea davidii* have been studied by previous generations (Kanehisa *et al*., 2004; Liu *et al*., 2020). The hard cuticle and large epidermal cells are both essential structural features for coping with drought, and the *H. davidii* is rich in medicinal properties, resisting inflammation, antioxidants, and inhibiting bacteria. Besides its medicinal value, *H. davidii* can also be used to make agricultural machines and furniture, and its tender leaves are rich in nutrition and can be used to make feed for livestock. While the codon bias of organelle genes was generally moderate, it was stronger in the chloroplast of *H. davidii* than in the mitochondria. The organelle genome's base makeup at the third codon position exhibits a pronounced A/T bias. Natural selection plays a major role in the formation of codon bias in organellar genes (Liu *et al*., 2020).

The normal growth and development of plants are significantly hampered by drought, salt, frost damage, plant diseases, insect pests, and other adverse environmental conditions. Drought, as a common globlal meteorological disaster with the highest frequency, the longest duration, and the widest area of impact, has always been one of the main natural disasters facing mankind (Dai, 2013). According to statistics, arid and semi-arid regions in the world account for one-third of the land area (Fang & Xiong, 2015). China is a country with a shortage of water resources, studies have shown that arid and semi-arid areas in China have accounted for 45% of the land area. This situation has brought many problems to the growth of plants and seriously affected the development of agriculture and forestry in China (Huang *et al*., 2017). Overly severe drought conditions can lead to loss of expansion pressure or even death of plant cells. Faced with the current situation of drought, plants also adopt a variety of ways to deal with it, such as entering dormancy or regulating their metabolic processes in the body (Seleiman *et al*., 2021), even formed a set of mature drought-resistant mechanisms to resist drought (Ramachandra *et al*., 2004).

After drought stress, the activities of nitrite reductase (NR) and glutamate synthase (GOGAT) in the leaves and roots of plants are decreased (Meng *et al*., 2016), the surface area of chloroplasts is increased, with a slight structural degradation., and the accumulation of carotenoid free radicals in chloroplasts of plants with strong tolerance was higher (Filek *et al*., 2016). People also evaluate the drought resistance of plants through the changes of osmoregulation substances, plasma membrane permeability, and enzyme protection systems (Fan *et al*., 2008).

Transcriptome sequencing (RNA-seq) is an efficient and economical method for quantitatively analyzing biological transcriptome data. It uses the new generation sequencing technology (second generation sequencing technology) to sequence the cDNA library of all RNAs reverse transcribed in a tissue or cell to obtain the sequence

information and expression information of almost all transcripts in a specific cell or tissue in a certain state (Henson, J. *et al*., 2012). Currently, it is widely used to study the response of plants to biotic and abiotic stresses.

In this study, we used PEG-6000 to simulate drought stress to investigate the changes of physiological indexes such as phenotype, antioxidant metabolism mechanism, osmoregulation mechanism and chlorophyll content of *Hemiptelea davidii* leaves under different PEG stress, and transcriptome sequencing analysis was conducted on leaves of *Hemiptelea davidii* under PEG stress, which laid the foundation for further exploration of physiological response and internal molecular mechanism of *Hemiptelea davidii* under drought stress, with a view to promoting drought-tolerant plants, developing arid areas, protecting species diversity and improving ecological habitat quality.

Material and Methods

Experimental materials and treatment: The experimental materials were the branches of the annual *Hemiptelea davidii* in the knowledge garden and forest farm of Northeast Forestry University, Harbin. The 12cm-15cm-long branch cutts of *Hemiptelea davidii* were cultured in the soil mixed with charcoal soil, vermiculite, and perlite in a ratio of 6:3:1. Plants with good growth and relatively consistent plant height were selected and transferred to light-sheltered conical flasks for slow seedlings. On the 8th day, 4 groups of PEG-6000 solution (0, 10%, 20%, 30%) with different concentrations were configured with MS nutrient solution to simulate drought treatment. Leaves were sampled from different treatments at 0h, 6h, 12h and 24h after drought stress for the determination of physiological and biochemical indices.

The seedlings of *Hemiptelea davidii*, were moved into the same amount of 30% PEG-6000 solution with MS solution as a solvent on the morning of the 8th day of delayed seedling, and three treatments were set up. The CK group was treated for 0 hour, and two experimental groups for 6 and 12 hours, respectively. The functional leaves of *Hemiptelea davidii* were collected for total RNA extraction. The sample names are: L01, L02, and L03 are three groups of CK, L04, L05, and L06 are three groups of stress samples for 6 hours; and L07, L08, and L09 are three groups of stress samples for 12 hours.

Physiological and biochemical index measurement methods: Soluble sugar (SS) content was determined by phenol method; Soluble protein content was determined by BCA protein content determination kit; the free proline content was determined by acid ninhydrin method; the activity of superoxide dismutase (SOD) was determined by the reduction method of NBT; Peroxidase (POD) activity was determined by guaiacol chromogenic method; catalase (CAT) activity was determined by UV spectrophotometry method; malondialdehyde (MDA) content was determined by thiobarbituric acid (TBA) method; the SPAD value of *Hemiptelea davidii* leaves was measured by SPAD chlorophyll meter.

Construction and sequencing of library: Total RNA was extracted from 9 sequencing samples in CK group and two experimental groups, respectively. After testing the purity, concentration and integrity of RNA extracted from *Hemiptelea davidii*, purified mRNA was obtained by the action of magnetic beads with Oligo (dT) on RNA samples, followed by the addition of fragmentation buffer for fragmentation. After the cDNA is purified, the terminal repair and linker are carried out; PCR was used to construct the library; Qubit 3.0 and Agilent 2100 were used to ensure the quality of the library. Finally, based on the technology of synthesis and sequencing, Illumina Hiseq high-throughput sequencing platform was used to sequence the qualified cDNA library.

Assembly of sequencing data and Unigene function annotation: Using Illumina Hiseq high-throughput sequencing platform to sequence the library, to obtain high-quality clean data. The length of Unigene and Transcript obtained by assembly was analyzed, and the Unigene was compared with seven databases of NR, Swiss-Prot, GO, COG, KOG, KEGG and eggNOG by using BLAST software. After predicting the sequences corresponding to the amino acids of Unigene, the annotation information of the stinging elm Unigene was obtained by comparing with Pfam database by using HMMER software.

Analysis of differentially expressed genes: Bowtie software was used to compare the Reads obtained by sequencing each leaf sample of *H. davidii* before and after PEG stress with the Unigene library, and RSEM was used to estimate the expression level. The expression abundance of the corresponding Unigene was expressed by the FPKM value. DESeq2 was used to analyze the differential expression among leaf sample sets of *H. davidii* under different treatments to obtain a set of differentially expressed genes between the two treatments. We used Fold Change (FC) and corrected significant pvalue (FDR) as the main references for differential gene screening, with False Discovery Rate (FDR)<0.01 and FC≥2 (FDR<0.01 and |log 2 FC|≥2) used as the screening criteria to obtain the final differentially expressed genes.

Analysis of differentially expressed genes GO, COG and KEGG: In this experiment, the differential genes were classified by GO, and the biological functions of differentially expressed genes are mainly analyzed from molecular function, cellular component, and biological process. COG annotation analysis was performed on differentially expressed genes in the experimen. Pathway enrichment of differentially expressed genes in leaves of *H. davidii* was analyzed by using KOBAS2.0 software, and the enrichment significance was calculated by Fisher.

Transcription factor analysis: The iTAK software was utilized to predict and analyze the transcription factor that were altered in *H. davidii* under PEG stress, and Hmmscan was used to search the HMM library to identify these transcription factors.

Data processing: Excel 2007, SPSS Statistics, sigmaplot 12.5 and Origin 2023 were used to process and analyze the experimental data. Excel 2007 was used for the entry and collation of raw data; SPSS Statistics was used for one-way ANOVA of the data and Duncan's method for multiple comparisons (Pallant, 2020); Origin 2023, sigmaplot12.5 and Excel 2007 were used for plotting.

Results

Effects of drought stress on osmoregulation substances in leaves of *Hemiptelea davidii*: Plants' osmoregulatory system is an important physiological process that allows them to endure environmental stress. To determine if *H. davidii* can withstand drought stress, the osmoregulation substances of *H. davidii* were evaluated during drought stress. Drought raised the concentration of osmotic regulating substances, which enhanced drought tolerance in *H. davidii*. As shown in (Fig. 1A) below, with the increase of drought duration and intensity, the soluble sugar content in the leaves of *H. davidii* generally shows an increasing state. The soluble sugar content was significantly higher than CK at 6h of stress at 20% and 30% PEG concentration, at 12h of stress at all three concentrations, and at 24h of stress at 30% PEG concentration. Under the stress of 30% PEG for 24h, the soluble sugar content reached the peak value of 7.47%, which was also the maximum value of soluble sugar content of *H. davidii* under different drought and concentration.

Changes of soluble protein content in leaves of *H. davidii* under PEG stress are shown in (Fig. 1B) below. At the drought of 6h, with the increase of PEG concentration, the content of soluble protein increased at first and then decreased. There was a significant difference between the content of soluble protein and CK. At the drought of 12h, with the increase of PEG concentration, the soluble protein content was decreased at first, then increased and then decreased. Only at the stress of 20% PEG, the content of soluble protein was significantly different from CK, which was 1.33 times that of CK. At the drought of 24h, with the increase of PEG concentration, the soluble protein content gradually increased, which was significantly higher than CK 1.25 times, 1.34 times and 1.49 times of CK, respectively.

The changes in free proline content of PEG-stressed *H. davidii* leaves are shown in (Fig. 1C) below. At 6h of stress, free proline content was increased with increasing PEG stress concentration, at 12h of stress, free proline content was increased, then decreased and then increased again, and at 24h of stress, free proline content was increased with increasing PEG stress concentration, and free proline content was significantly higher at different drought concentrations and times than at CK. The content of free proline in the leaves of *H. davidii* reached the maximum value of 77.1 $\mu g \cdot g^{-1}$ at 24h of 30% PEG.

Effect of drought stress on malondialdehyde content in leaves of *Hemiptelea davidii*: Malondialdehyde (MDA), a byproduct of cell membrane lipid peroxidation, can disrupt biofilm structure and its level can reflect plant drought tolerance. Changes of MDA content in leaves of *H. davidii* under PEG stress are shown in (Fig. 1D) below. At the drought of 6h, with the increase of PEG concentration, the content of MDA first increased, then decreased and then increased, and the content of MDA in all concentrations was significantly different from CK. At the drought of 12h, with the increase of PEG concentration, the content of MDA was increased and then decreased, among which the MDA content only under 20% PEG stress was significantly different from CK. At the drought of 24h, with the increase of PEG concentration, the content of MDA also increased and then decreased. Only under the stress of 10% PEG, the content of MDA was significantly different from CK. The maximum value of MDA in the experiment appeared under 20% PEG stress at 12h, which was 3.58 $\mu mol \cdot g^{-1}$.

Effect of drought stress on antioxidant enzymes in leaves of *Hemiptelea davidii*: In comparison to CK, the activities of POD, SOD, and CAT in *H. davidii* seedlings were increased initially and subsequently dropped as drought and temperature increased. The change of SOD activity in leaves of *H. davidii* under PEG stress is shown in (Fig. 1E) below. At the drought of 6h, with the increase of PEG concentration, the SOD activity generally showed an upward trend, and under the stress of different concentrations, the SOD activity increased significantly compared with CK. At the drought of 12h, with the increase of PEG concentration, the SOD activity increased, then decreased and then increased. Compared with CK, the SOD activity at different PEG concentrations was significantly different, and the SOD activity at 30% PEG reached the maximum of 175.65 $U \cdot g^{-1}$, which was 3.88 times that of CK. At 24 hours of stress, with the increase of PEG concentration, SOD activity first increased and then decreased.

The changes of POD activity in leaves of *H. davidii* under PEG stress are shown in (Fig. 1F) below. Under the drought of 6h, with the increase of PEG concentration, the POD activity generally showed an upward trend. Compared with CK, the POD activity under different concentrations of stress was significantly increased. Under the drought of 12h, with the increase of PEG concentration, the activity of POD increased, then decreased and then increased. Compared with CK, the activity of POD at all PEG concentrations increased significantly. At the drought of 24h, with the increase of PEG concentration, the POD activity increased at first. Then it decreased, which was significantly different from CK and reached the maximum value of 65.03 $U \cdot g^{-1} \cdot min^{-1}$ at 10% PEG concentration, and then decreased with the increase of PEG concentration, and reached the minimum at 30% PEG.

The changes of CAT activity in leaves of *H. davidii* under PEG stress are shown in (Fig. 1G) below. At the drought of 6h, the CAT activity also increased with the increase of PEG concentration, and there were significant differences between CAT activity and CK. At the drought of 12h, with the increase of PEG concentration, the activity of CAT was increased and then decreased, and there were significant differences between CAT and CK. At the drought of 24h, CAT activity tended to increase and then decreased with increasing PEG concentration, and was 1.83, 7.33 and 3.59 times higher than that of CK at all stress levels ($p<0.05$), respectively.

Effects of drought stress on chlorophyll content in leaves of *Hemiptelea davidii*: In general, drought stress causes water loss in plant leaves and affects chlorophyll synthesis in plants. With the prolongation of PEG drought and the increase of concentration, the SPAD value in the leaves showed a downward trend and reached the minimum of 12.4 when 30% PEG was applied for 24 h. Moreover, at 12h and 24h of stress, compared to 10%PEG stress concentrations, the SPAD values of 30% PEG were decreased significantly ($p<0.05$), with a decrease of 28% and 24%, respectively (Fig. 1H). It could be seen that the increase of PEG stress could lead to the decrease of chlorophyll content in leaves of *H. davidii*, which might further affect the photosynthesis of plants.

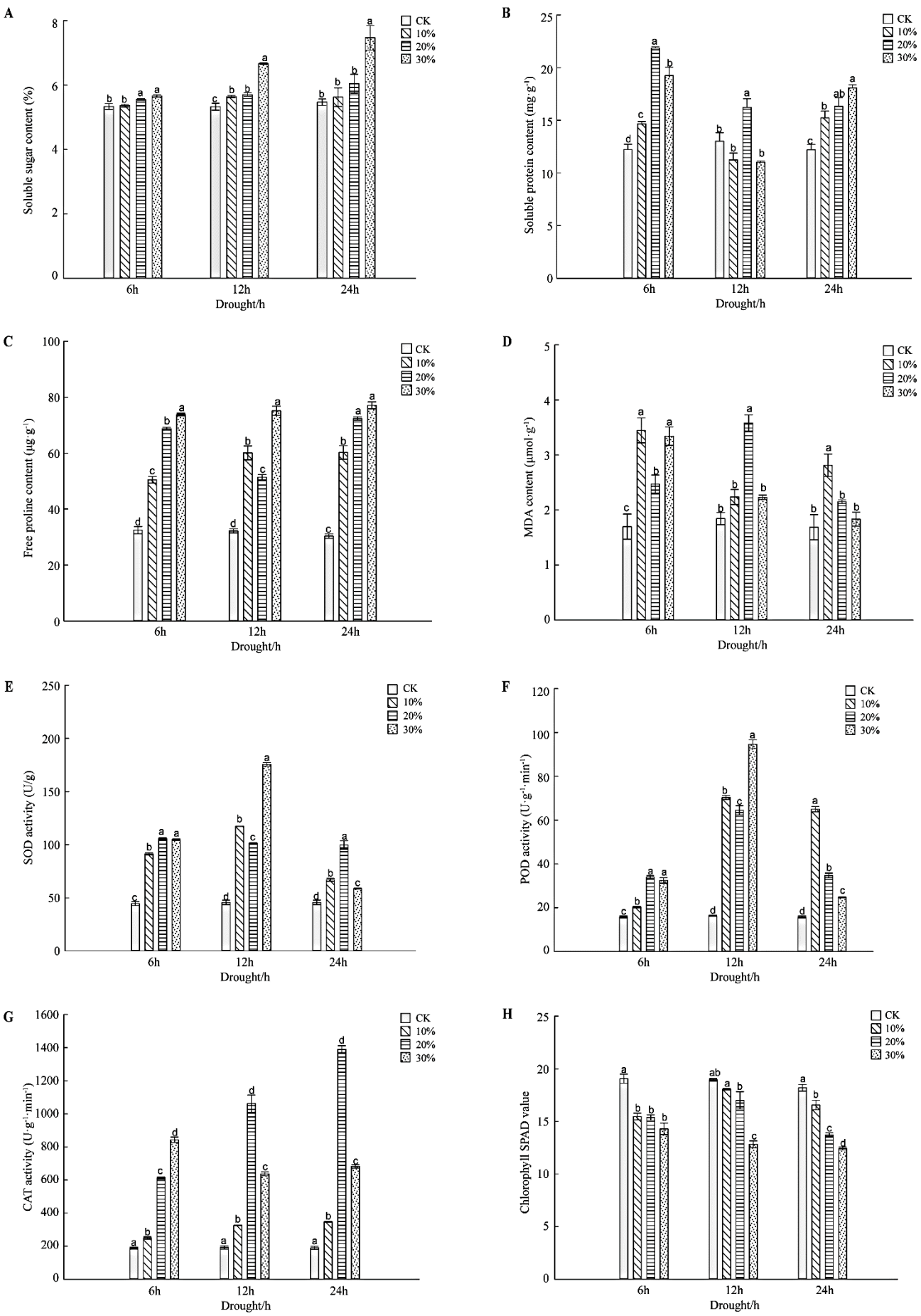

Fig. 1. Effects of Drought on Physiological and Biochemical Indexes in Leaves of *Hemiptelea davidii*.

(A) Soluble sugar content. (B) Soluble protein content. (C) Free proline content. (D) MDA content. (E) SOD activity. (F) POD activity. (G) CAT activity. (H) Chlorophyll SPAD value.

Note: Error lines in the graph indicate the size of the standard deviation, different letters represent significant differences between treatments (p<0.05) and the same letter represents non-significant differences (p>0.05).

Correlation analysis of physiological indexes of *Hemiptelea davidii*: The correlation analysis of physiological indexes of *H. davidii* under different PEG stress concentrations and durations is shown in the following (Fig. 2). The results showed that the chlorophyll content in leaves of *H. davidii* at 6h, 12h and 24h of PEG stress had a very significant negative correlation with PEG concentration (p<0.01). The contents of osmoregulation substances SS and Pro in *H. davidii* leaves under three stress times were highly significantly positively correlated with PEG concentration (p<0.01). MDA content showed a very significant negative correlation with it at 6h; for antioxidant enzyme activity, PEG concentration exhibited a very significant positive correlation with SOD and POD activities at 6h and 12h, but no correlation at 24h stress (p>0.05), and a very significant positive correlation with CAT activity at 6h and12h stress (p<0.05). The results showed that under a certain stress time, with the increase of PEG concentration, the chlorophyll content in *H. davidii* leaves was decreased, MDA accumulated, the contents of osmoregulation substances SS, SP and Pro in leaves increased, and the activities of SOD, POD and CAT were increased significantly to resist PEG stress.

(A) Correlation analysis of physiological indicators of *H davidii* under different concentrations of PEG stress for 6h. (B) Correlation analysis of physiological indicators of *H. davidii* under different concentrations of PEG stress for 12h. (C) Correlation analysis of physiological indicators of *H. davidii* under different concentrations of PEG stress for 24h.

Note: * indicates significant correlation at 0.05 level, * * indicates significant correlation at 0.01 level.

Sequencing data quality assessment: In this study, a total of 9 *H. davidii* samples were sequenced at three times (0h, 6h and 12h) under 30% PEG-6000 stress, with three biological replicates at each time point (Table 1). After data filtering of the raw data and sequencing quality control, a total of 66.60 Gb Clean Data was obtained, with each sample Clean Date greater than 6.44 Gb. The Read number in each *Hemiptelea davidii* sample was greater than 21.52 million, the GC content was between 45.33% and 45.74%, and the base percentage of Q30 in each *H. davidii* sample was not less than 93.65%, indicating that the base recognition accuracy was high, and the sequencing result quality was good. The sequencing result of the transcriptome met the quality requirements for subsequent assembly.

Transcriptome sequence assemble and comparison with sequencing data: After assembly with Trinity software, a total of 204,453 Transcript and 54598 Unigenes were obtained (Table 2). The average length of Transcript is 1904.09bp, and that of Unigene is 941.14bp, of which 7459 Unigenes are longer than 2000, accounting for 13.66% of all Unigenes. Unigenes' N50 length is 1815bp, while the N50 length of Transcript is 2695bp, with high assembly integrity, which meets the needs of subsequent functional analysis of genes.

Table 1. Sequencing data evaluation statistics.

Sample	Read number	Base number	GC content	%≥Q30
L01	25,904,148	7,750,987,990	45.47%	94.89%
L02	21,523,398	6,444,460,348	45.55%	94.88%
L03	22,591,722	6,759,266,024	45.61%	95.31%
L04	26,909,609	8,052,534,130	45.56%	94.21%
L05	25,689,028	7,682,716,462	45.74%	94.52%
L06	25,410,124	7,599,403,398	45.54%	94.33%
L07	24,518,777	7,330,311,652	45.33%	94.17%
L08	25,345,659	7,588,076,072	45.73%	93.78%
L09	24,698,523	7,390,648,080	45.47%	93.65%

Table 2. Unigene assembly information of *Hemiptelea davidii* seedling leaves.

Length Range	Transcript	Unigene
200-300	19,174(9.38%)	16,290(29.84%)
300-500	17,639(8.63%)	12,058(22.09%)
500-1000	28,206(13.80%)	10,542(19.31%)
1000-2000	56,611(27.69%)	8,249(15.11%)
2000+	82,823(40.51%)	7,459(13.66%)
Total Number	204,453	54,598
Total Length	389,296,192	51,384,228
N50 Length	2,695	1,815
Mean Length	1904.09	941.14

Note: Total Number: denotes the total number of Unigene assembled; Total Length: denotes the total length of Unigene assembled; N50 Length denotes the length of N50 of Unigene; Mean Length: denotes the average length of Unigene.

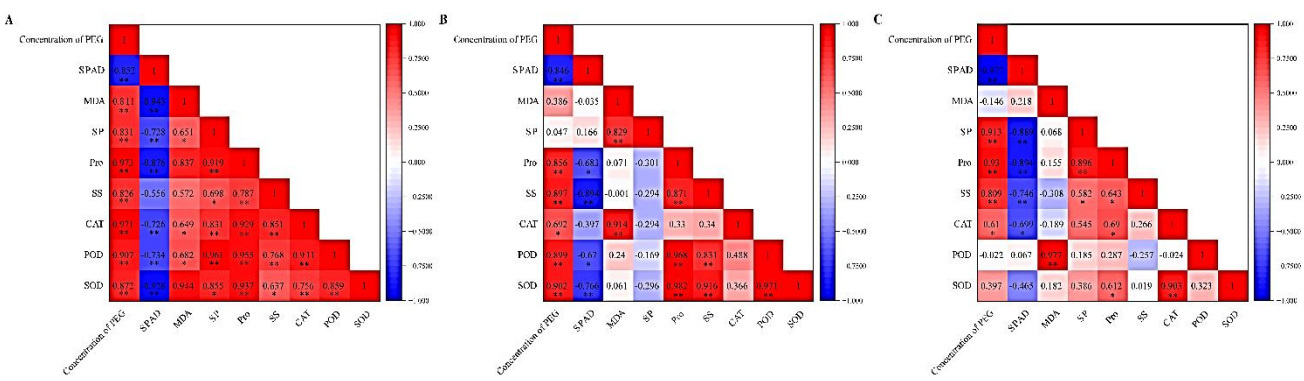

Fig. 2. Correlation analysis of physiological indexes of *Hemiptelea davidii*.

Functional annotation of Unigene genes: The Unigene sequence of *H. davidii* was compared with NR, Swiss-Prot, GO, COG, KOG, eggNOG, and KEGG databases using BLAST software (Altschul *et al*., 1997). As shown in (Table 3) below, the results showed that among the 54598 Unigenes in the leaves of *H. davidii* under PEG stress, 30791 were annotated, accounting for 56.4%, among which the largest number of Unigenes were annotated to the eggNOG database, with 28186 (51.6%), and the least Unigenes annotated to the COG database were 10179 (18%).

Table 3. Unigenes function annotation result statistics.

Annotated databases	Annotated number	300<=length	length>=1000
NR	27831	10336	14421
SwissProt	19590	5880	10747
KEGG	12333	4097	6033
COG	10179	2736	5923
KOG	19036	6339	9345
eggNOG	28186	9265	14087
GO	18338	5441	9612
Pfam	22025	6900	12155
All	30791	10366	14421

Analysis on overall distribution of gene expression in *Hemiptelea davidii* samples: The value of Fragments Per Kilobase of transcript per Million mapped reads (FPKM) represents the expression abundance of Unigene and indicates the number of reads per kilobase length per million bases from a gene compared to a gene (Trapnell *et al*., 2010). It could be seen that the gene expression levels of different *H. davidii* samples were slightly different. Most of the gene expression levels in the 9 samples were within the range of 0.1-100, and only a few genes were highly expressed with the expression level greater than 10^4 (Figs. 3A and B).

Statistical analysis of differentially expressed genes: Differential expression among sample groups was performed by DESeq2, and the sample combinations of L01, L02, and L03 were named as w1, the combinations of L04, L05, and L06 as w2, and the combinations of L07, L08, and L09 as w3. As shown in (Fig. 4A) 620 DEGs were found in the w1_w2 sample, of which 544 were upregulated and 76 were downregulated. Among the 3803 DEGs in the w1_w3 sample, 1852 were upregulated and 1951 was downregulated. The clustering pattern diagram of DEGs between w1_w2 and w1_w3 *H. davidii* samples is as follows, DEGs with the same or similar expression behaviors are clustered. There were significant differences in gene expression patterns among the *H. davidii* samples treated with w1, w2 and w3, and the gene expression difference in w1_w3 *H. davidii* sample was greater than those in w1_w2. However, the gene expression was similar among the three replicates within each of the three groups of different treated *H. davidii* samples (Figs. 4B and C). The Volcano Plot of DEGs between w1_w2 and w1_w3 *H. davidii* samples showed that w1_w3 had more DEGs expressed compared with w1_w2, suggesting that with the prolongation of drought, more complex gene regulation might be involved (Figs. 4D and E).

GO analysis of differentially expressed genes: The GO classification of DEGs could be divided into three categories: cellular component, molecular function, and biological process. In the w1_w2 sample combination, 411 DEGs obtained GO annotation, involving 40 groups in three categories, where cellular component and molecular function each involved 12 functional groups, among which 12 functional groups were involved in cellular component and molecular function each, and 16 functional groups were involved in biological process (Fig. 5A). DEGs among w1_w2 *H. davidii* samples were mainly enriched in cell, cell part, membrane part, membrane, organelle, and other functions in cell components. In biological processes, DEGs were mainly enriched in metabolic process, cellular process, single-organism process, and other functions. In terms of molecular functions, DEGs were mainly enriched in catalytic activity, binding, and transportation activity. Among the DEGs in the w1_w3 sample combination, 2475 DEGs obtained GO annotations, involving 44 groups in 3 categories, among which 16 functional groups were involved in each of cellular component and biological process, and 12 functional groups were involved in molecular function (Fig. 5B). DEGs among w1_w3 *H. davidii* samples were mainly enriched in cell, cell part, membrane part, membrane, organelle, and other functions in cell components. In biological processes, DEGs were mainly enriched in metabolic process, cellular process, single-organism process, biological regulation, localization, response to stimulus and other functions. Among the molecular functions, DEGs were mainly enriched in catalytic activity, binding, transporter activity, nucleic acid binding transcription factor activity, and other functions. It could be found that these DEGs might play an important role in regulating some growth, development, and physiological regulation processes of *H. davidii* under PEG stress.

COG analysis of differentially expressed genes: Carry out COG annotation analysis on DEGs. In w1_w2, 225 DEGs obtained COG annotation, involving 23 terms, of which the top three annotated terms were signal transduction mechanisms, carbohydrate transport and metabolism, and lipid transport and metabolism, the number of annotated DEGs in this function was 39, 38, and 21, respectively (Fig. 6A). In w1_w3, 1349 DEGs obtained COG annotation, involving 24 terms. The five terms with the most DEGs were carbohydrate transport and metabolism, general function prediction only, signal transduction mechanisms, lipid transport and metabolism, post-translational modification, protein turn over, chaperones, with 190, 157, 144, 128, and 127 DEGs, respectively (Fig. 6B).

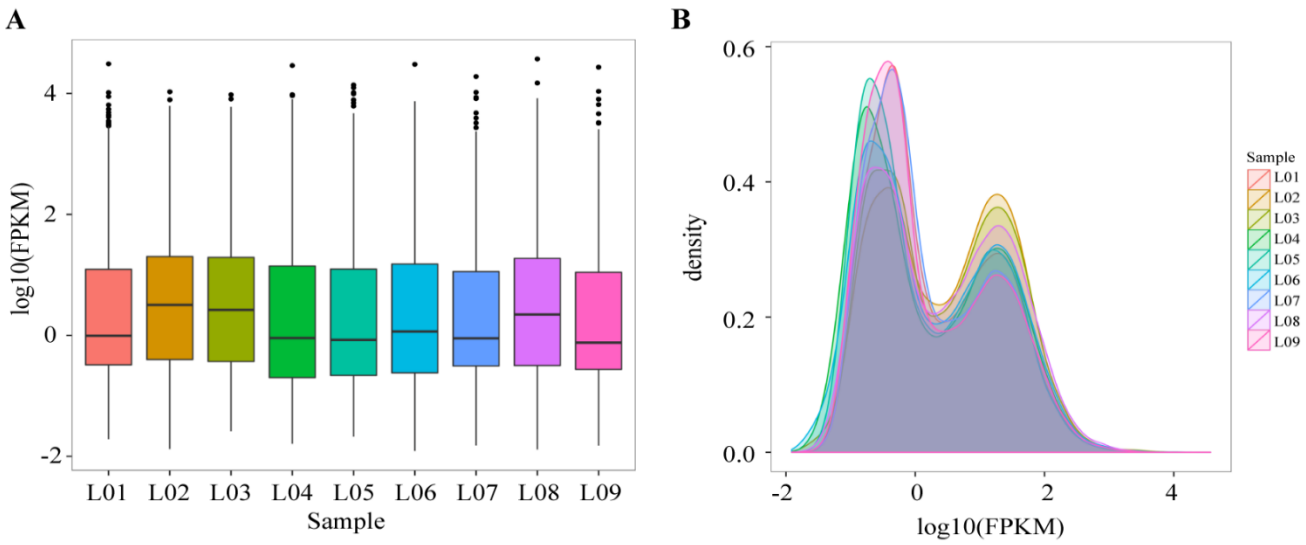

Fig. 3. Overall distribution of sample gene expression. (A) FPKM boxplot of each *Hemiptelea davidii* sample. (B) Comparative diagram of FPKM density distribution of each *Hemiptelea davidii* sample.

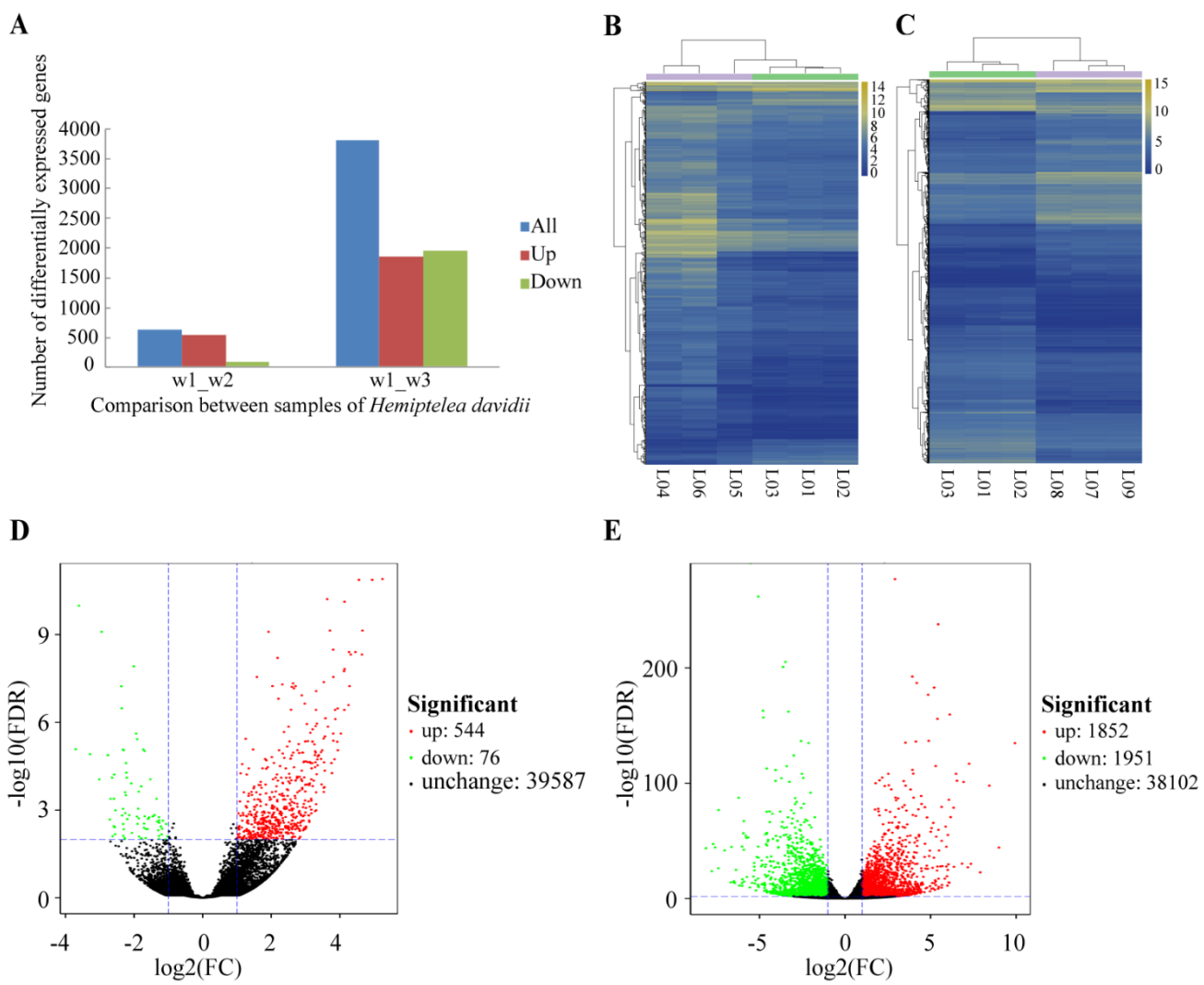

Fig. 4. Statistical diagram of DEGs among *Hemiptelea davidii* samples. (A) Statistical diagram of DEGs between w1_w2 and w1_w3 *H. davidii* samples. (B) Cluster analysis of DEGs in w1_w2 comparison group. (C) Cluster analysis of DEGs in w1_w3 comparison group. Color representing the pair value of gene expression in samples FPKM with 2 as the base, blue is the gene with low expression, yellow is the gene with high expression. (D) Volcano map of DEGs in w1_w2 *H. davidii* sample. (E) Volcano map of DEGs in w1_w3 *H. davidii* sample. Each dot in the figure signifies a particular DEG. The red dot shows upregulated DEGs, the green dot indicates downregulated DEGs, and the dark grey dot is a nonsignificant differential gene.

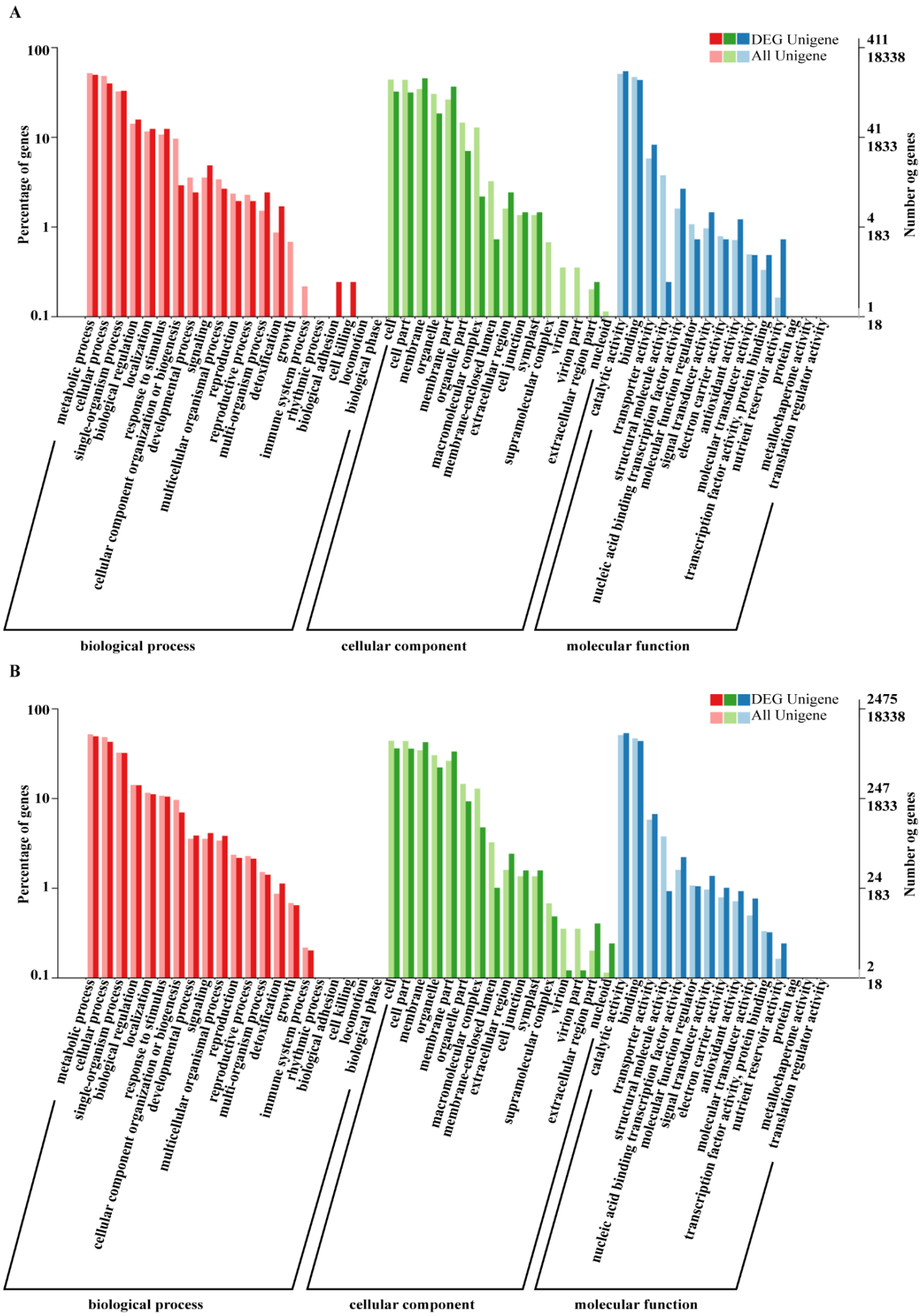

Fig. 5. Gene Ontology (GO) analysis of DEGs. (A) Histogram of GO statistics for w1_w2 DEGs. (B) Histogram of GO statistics for w1_w3 DEGs.

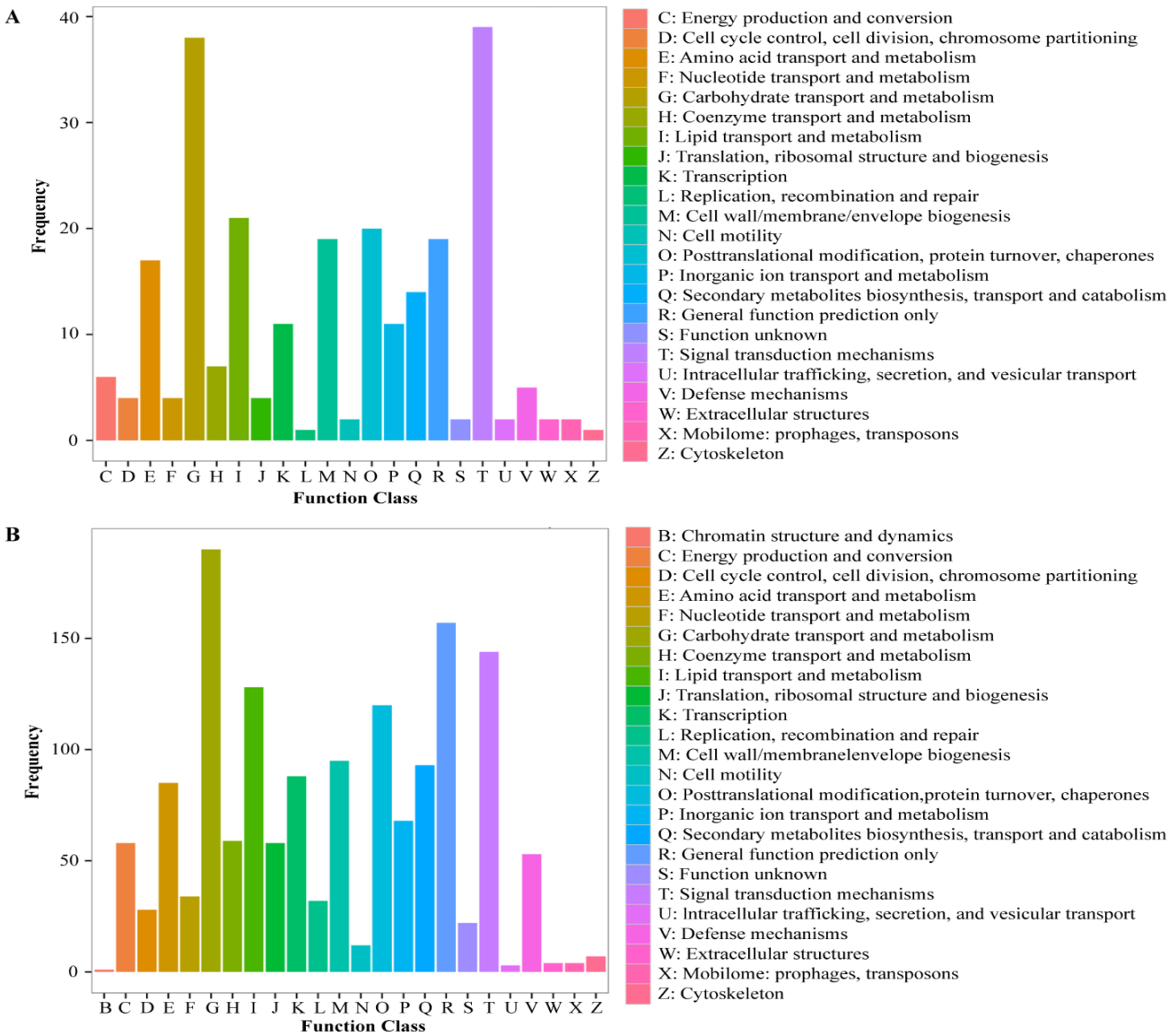

Fig. 6. Cluster of Orthologous Groups of proteins (COG) analysis of DEGs. (A) COG classification of DEGs between w1_w2 samples. (B) COG classification of DEGs between w1_w3 samples.

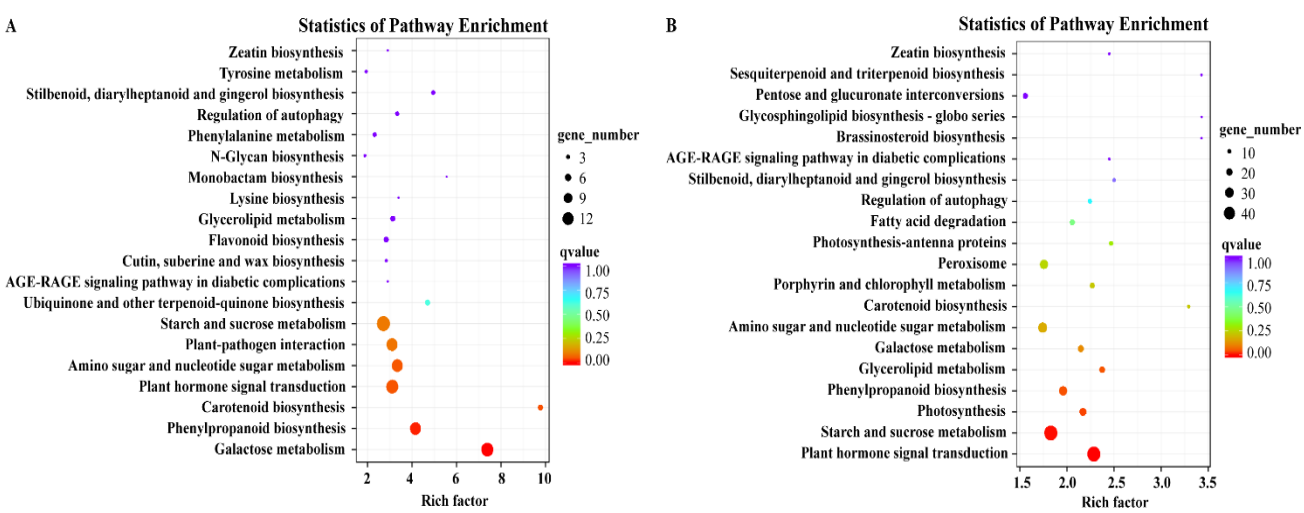

Fig. 7. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway of DEGs. (A) Scattering plot of KEGG enrichment of DEGs between w1_w2 samples. (B) Scattering plot of KEGG enrichment of DEGs between w1_w3 samples. The rich factor in the horizontal axis is the size of the point, which represents the number of DEGs, and the color of the dot represents the q value. The darker the circle color in the diagram was, the more significant the enrichment degree of differentially expressed genes was.

KEGG enrichment analysis of differentially expressed genes: In the w1_w2 group of DEGs samples, 589 DEGs were annotated into 61 metabolic pathways, the top 5 pathways with the highest enrichments of DEGs were starch and sucrose metabolism, plant hormone signal transduction, plant-pathogen interaction, amino sugar and nucleotide sugar metabolism, and phenylpropanoid biosynthesis. Among them, the DEGs in starch and sucrose metabolism were the most abundant with 12 DEGs (Fig. 7A). In the w1_w3 group of DEGs samples, 1209 DEGs were annotated into 120 metabolic pathways. The top 5 pathways with significant enrichment were starch and sucrose metabolism, plant hormone signal transduction, amino sugar and nucleotide sugar metabolism, peroxisome, phenylpropanoid biosynthesis. The pathways with the most DEGs annotated were plant hormone signal transduction and starch and sucrose metabolism, with 48 DEGs annotated on both pathways (Fig. 7B). Among them, the DEGs in both w1_w2 and w1_w3 comparison groups were involved and significantly enriched were starch and sucrose metabolism, plant hormone signal transduction and galactose metabolism. These metabolic pathways might play an important role in the response of *H. davidii* to PEG stress.

Partial metabolic pathway analysis: Several metabolic pathways with high significant enrichment of differential genes between w1_w2 and w1_w3 samples in the transcription group of *H. davidii* under PEG stress were analyzed. First, in w1_w2, drought stress upregulated the genes of 1-phosphate uridine acyltransferase, UDP-glucose 4-epimerase and inositol 3-α-galactosyltransferase in the galactose metabolic pathway as shown in (Fig. 8A) below. The conversion of UDP-glucose to galactitol was affected, and the gene of 6- phosphofructokinase 1 (PFK1) was upregulated, which catalyzed the phosphorylation reaction of D-tagatose-6P and α-D-glucose. In addition, the stress also upregulated the expression of raffinose synthase, stachyose synthase, and β-fructose furanosidase, catalyzing the conversion of galactitol, and the reaction caused the content of products raffinose, stachyose, manninotriose and melibiose to increase.

In the phenylpropane biosynthesis pathway (Fig. 8B), under PEG stress, genes such as Cinnamyl-CoA reductase (CCR), oxalate O-hydroxycinnamoyl transferase, caffeic acid 3-O-methyltransferase, trans-cinnamic acid 4-monooxygenase, and caffeoyl coenzyme-A-O-methyltransferase were upregulated. These enzymes convert trans-cinnamic acid into cinnamaldehyde and sinapic acid, then catalyze the formation of products such as sinapoyl alcohol and coniferyl alcohol; at the same time, partial peroxidase gene was upregulated. It catalyzes the synthesis of products such as guaiacyl lignin and syringin to protect plants from external stresses such as drought.

Astaxanthin is a β -carotene derivative with strong oxidation, while β-carotene hydroxylase (CrtZ) and β-carotene ketolase (CrtW) were the key enzymes for astaxanthin synthesis. In carotenoid synthesis pathway (Fig. 9C), after *H. davidii* suffered from PEG stress, the upregulation of CrtZ enzyme gene and CrtW acted together to catalyze the production of adonixanthin and astaxanthin; at the same time, the upregulation of the enzyme gene also catalyzed the synthesis of lutein. Violaxanthin is a carotenoid, in this experiment, drought stress upregulated the 9-cis-epoxycarotenoid dioxygenase (NCED) gene and catalyzed the conversion of violaxanthin to xanthoxin.

Secondly, in w1_w3, the metabolic pathways of starch and sucrose metabolism under drought stress were shown in (Fig. 9A). The stress upregulated hexokinase, fructokinase and β-fructose furanosidase , which catalyzed the decomposition of sucrose. At the same time, some genes of glucose-1-phosphoadenyl transferase (glgC), β-glucosidase (bglX) and endoglucanase were upregulated, while the gene of endoglucanase 1,3-β- glucosidase (GN1_2_3) was downregulated, thus affecting the D-glucose synthesis pathway. In addition, α-amylase and β-amylase genes were upregulated to convert cyclodextrin to maltose.

The plant hormone signal transduction pathways under drought stress are shown in (Fig. 9B) below. Among them, abscisic acid (ABA) plays an important role in plants' response to drought stress. The increase of ABA under water stress can promote the accumulation of proline to maintain the stability of cell membrane structure. In addition, ABA can also induce the expression of LEA gene and form LEA protein, a macromolecule with the function of protecting organisms, which is able to maintain a specific cell structure and protect plants from the damage caused by drought stress. When plants were subjected to drought stress, a large quantity of ABA was induced to produce, it combined with the receptor PYR/PAL and interacted with PP2C to inhibit its activity, and then the inhibition of PP2C on the downregulated protein kinase SnRK2 was relieved. These protein kinases phosphorylate downstream transcription factors or ion channels located in the plasma membrane to initiate the ABA signaling response, and subsequently regulate the expression of drought-related genes or promotes stomatal closure to assist plants to withstand drought stress (Wang *et al*., 2019). However, a clade B member of PP2C negatively regulated plant drought tolerance through dephosphorylation of the MAPK signaling pathway in ABA-independent way, which negatively regulated ABA signaling (Lu *et al*., 2022). In this study, it was found that under PEG stress, the expression of PYR/PAL, the receptor of ABA, was inhibited, and the expression of PP2C enzyme gene was upregulated, but the reaction ultimately upregulated ABF, activating the expression of genes responsive to ABA signals to protect plants from dehydration to resist drought stress. Gibberellin (GA) also plays a significant role in the hormone regulation of plants responding to drought stress. In the experiment, under PEG stress, the gene expression of GID1 protein, the receptor of GA, was upregulated, and GAs combined with GID1 receptor and promoted the degradation of DELLA protein to mitigate the inhibition effect of PEG stress on plant growth.

As shown in (Fig. 9C), under drought stress, the expression levels of some genes involved in the photosynthesis pathway changed. There were 7 genes involved Photosystem II, of which two genes were upregulated (*PsbO* and *PsbR*), and five genes were downregulated (*PsbP*, *PsbQ*, *PsbW*, *Psb27* and *Psb28*). The six genes involved in Photosystem I were *PsbD*, *PsbE*, *PsbF*, *PsbH*, *PsbK* and *PsbL*, and all of them were downregulated. The three genes involved in type F ATPase were *ATPF1G*, *ATPF1D* and *ATPF0B*, all of which were downregulated. The genes related to photosynthethic electron transport were also downregulated, as *PetE*, *PetF*, and *PetH*. Thus, the expression levels of multiple genes involved in photosynthesis were changed and most genes were downregulated under drought stress, suggesting that PEG stress might have a significant impact on photosynthesis of *H. davidii*.

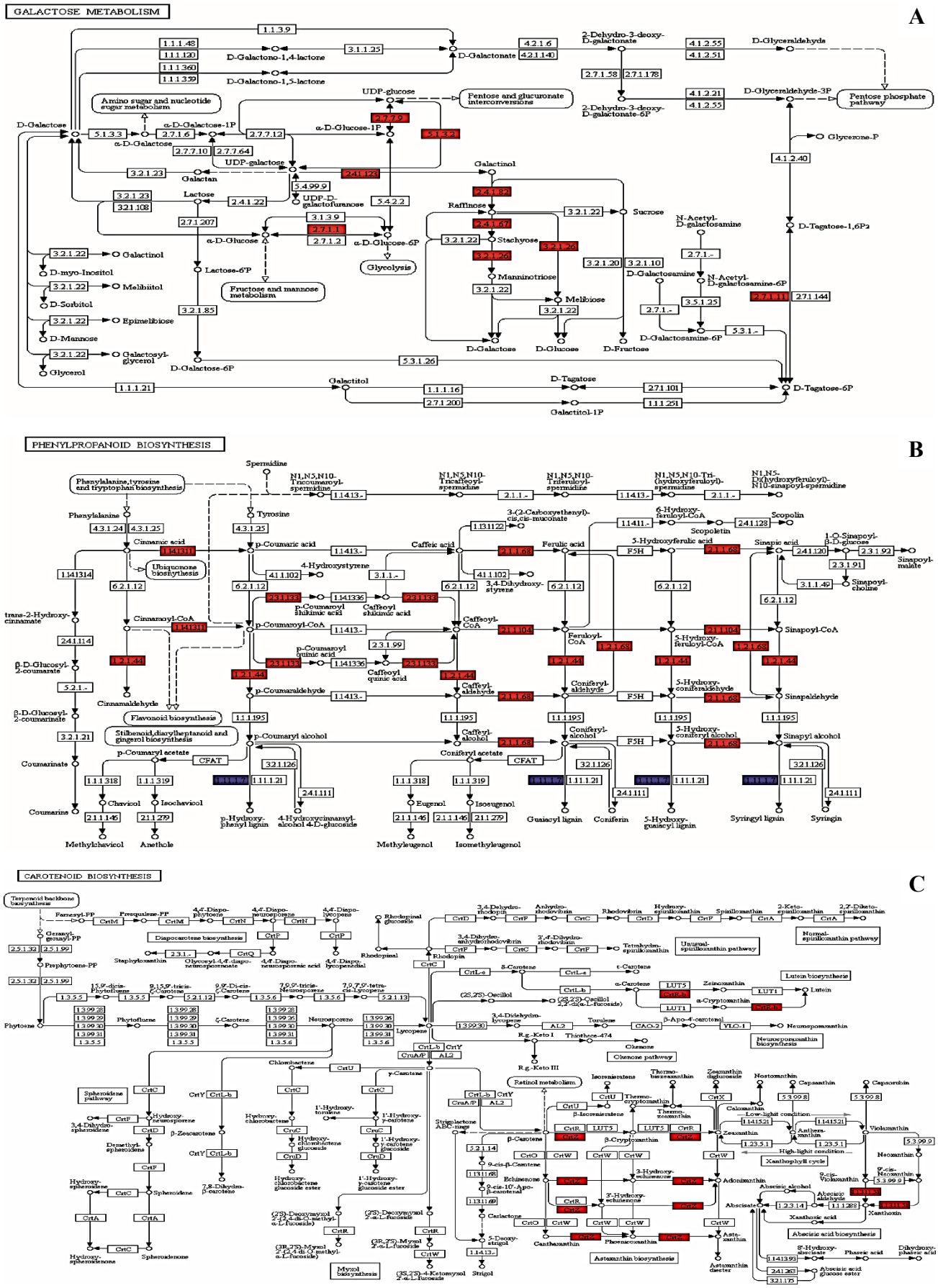

Fig. 8. KEGG metabolic pathway map of some differential genes in w1_w2. (A) Galactose metabolic pathway. (B) Phenylpropane biosynthesis pathway. (C) Carotenoid synthesis pathway.

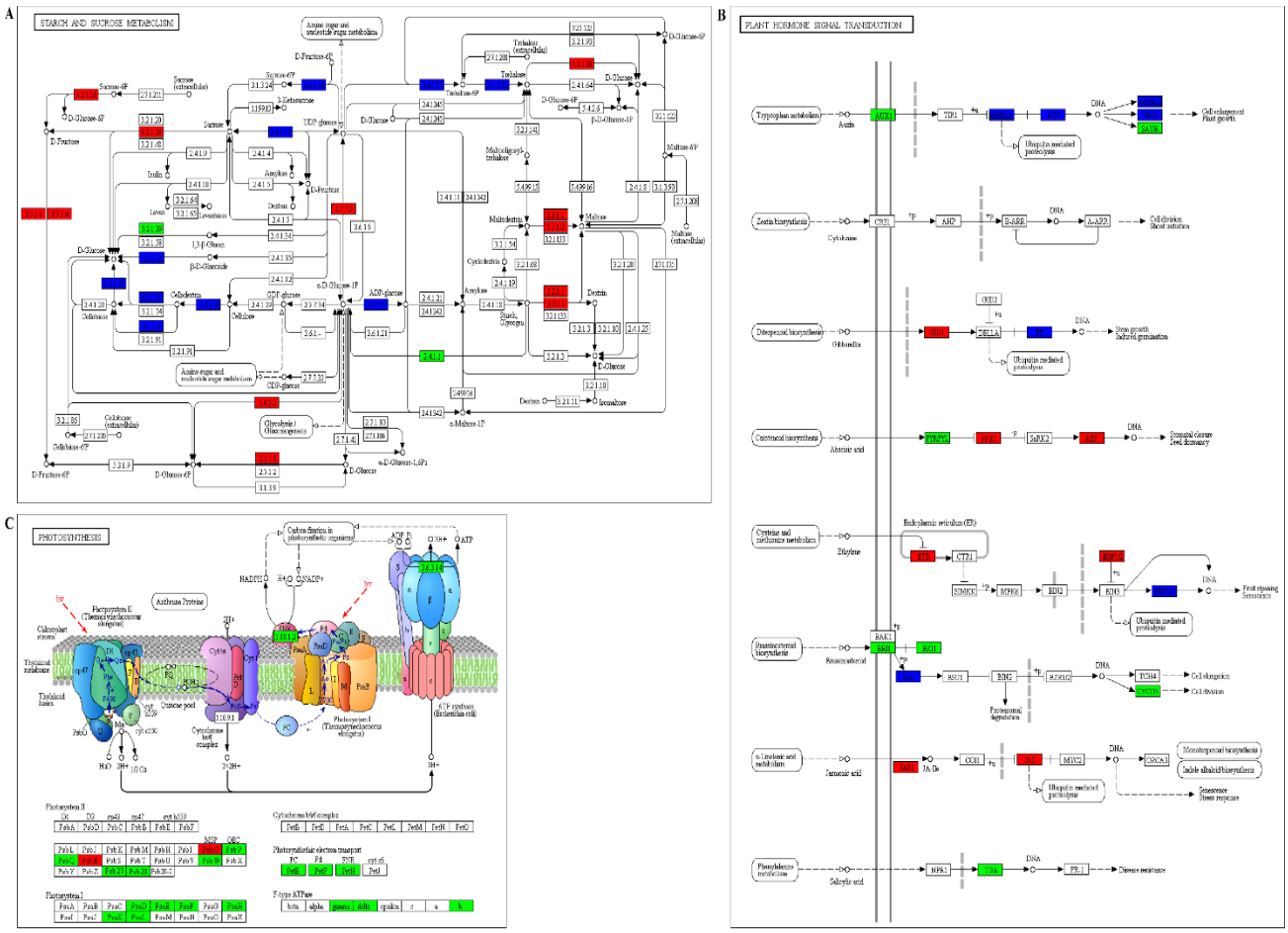

Fig. 9. KEGG metabolic pathway map of some differential genes in w1_w3. (A) Starch and sucrose metabolism pathway. (B) Plant hormone signal transduction pathway. (C) Photosynthesis pathway.

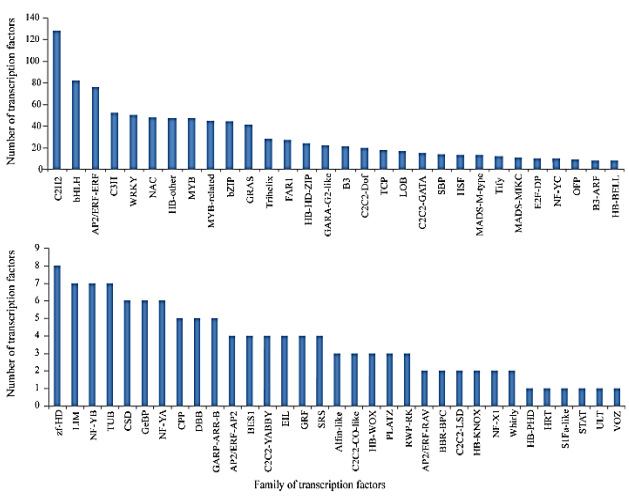

Fig. 10. Distribution of transcription factors.

Transcription factor analysis: When subjected to drought stress, plants can resist stress through the regulation of transcription factors in vivo. During the analysis of transcription factors of *H. davidii* in response to drought stress, a total of 1087 transcription factor genes were identified, involving 63 transcription factor families (Fig. 10). The top 10 families involved in transcription factors were C2H2, bHLH, AP2/ERF-ERF, C3H, WRKY, NAC, HB-other, MYB, MYB-related and bZIP. The C2H2 family contains the most transcription factors, involving 128 transcription factor genes, and C2H2-type zinc finger protein genes such as *c15220.graph_c0* were found in the family, which played an important role in assisting *H. davidii* to resist PEG stress. There are 82 transcription factors involved in bHLH family, and PEG stress makes some transcription factors of this family upregulated or downregulated, among which the upregulated expression of genes such as *c32652.graph_c0* of bHLH family was found in gibberellin signaling pathway. Transcription factor families such as AP2/ERF-ERF and bZIP have also been confirmed to play a role in plant hormone transduction pathway. In bZIP family, the up regulation of *c36955.graph_c0* gene was found in ABA signaling pathway, and the downregulation of *c35286.graph_c1* gene in salicylic acid signaling pathway was also found. In addition, the downregulated expression of transcription factor gene *c38203.graph_c0* related to ethylene response was also found in AP2/ERF-ERF family. These transcription factor genes may help *H. davidii* to resist drought stress by regulating plant hormone transduction pathway.

Discussion

Effect of PEG-6000 drought stress on osmoregulation substances in leaves of *Hemiptelea davidii*: Under drought stress, plants can actively accumulate cytosol through their osmoregulation system and secrete osmoregulation substances such as proline, soluble sugar,

soluble protein, etc., keep a certain cell swelling pressure, prevent plants from losing water, maintain plant morphology, and enable physiological activities such as photosynthesis to be carried out normally, so that plants can grow normally (Benkeblia, 2022).

In this experiment, the content of soluble sugar and proline in leaves of *H. davidii* showed an overall upward trend with the increase of stress duration and concentration. The content of soluble sugar and proline in *H. davidii* gradually increased under PEG stress, which could maintain the osmotic balance of plant cells and enhance the drought resistance of *H. davidii* seedlings, which was related to the study that soybean accumulated a large amount of Pro and increased its SS content in vivo to response to drought stress (Zhou *et al*., 2022). The concentration of soluble protein in leaves of *H. davidii* accumulated continuously at the early stage of stress (6h), decreased at the middle of stress (12h) and increased at the later stage (24h), which indicated that insoluble protein in plants was changed into soluble protein at the early stage of stress treatment to enhance osmoregulation capacity to resist drought. While at the middle stage of stress treatment, the activity of protease was increased to accelerate its hydrolysis, and drought caused by inhibition of RNA transcription and translation, decrease in protein synthesis, and increase in free amino acids (including increased Pro). Under severe soil drought stress, the physiological metabolism of cells in plants may be disturbed, and a large number of bound proteins may be degraded. It is generally believed that osmotic adjustment occurs in the case of light or moderate water stress. When water stress is very serious, osmotic adjustment capacity is weakened or lost (Kozlowski, 1982). Therefore, it can be concluded that soluble protein may not be the osmoregulation substance for *H. davidii* to obtain osmoregulation ability.

Effects of PEG-6000 drought stress on MDA content in leaves of *Hemiptelea davidii*: When plants are subjected to drought stress, free radicals rapidly accumulated, membrane lipid peroxidation level increased, and plant cell membrane was damaged, which increases the MDA content in cells (Mihaljevic *et al*., 2021). Therefore, MDA content can reflect the drought resistance of plants.

In this stress experiment, MDA content of *H. davidii* seedlings showed an overall increasing change state with the increase of PEG concentration at 6h of stress. The MDA content increased under mild and moderate PEG stress and decreased under severe stress when the stress time was 12 h. When the stress duration reached the longest of 24h, MDA content was increased under mild stress, and the trend of decline began under moderate stress, but the content was still higher than CK. The results showed that membrane lipid peroxidation caused by the deepening of drought stress led to the accumulation of MDA. However, with the increase of stress time and concentration, the activity of antioxidant enzyme was enhanced, most of the reactive oxygen species were cleared, and MDA content was decreased. However, excessive accumulation of reactive oxygen species under severe and long-term drought stress caused certain damage to the plant defense system, and the content of MDA was still higher than CK, which was similar to the findings of *Pandanus amaryllifolius* (Amnan *et al*., 2022).

Effects of PEG-6000 drought stress on protective enzyme system in leaves of *Hemiptelea davidii*: When plants are subjected to drought stress, the original dynamic balance of "production-removal" of reactive oxygen species in plants are distrupted, and reactive oxygen species in plants accumulate in large quantities. At this time, the reactive oxygen system in plants is stimulated to continuously increase the activities of certain antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD) and peroxidase (POD). The synergistic effect of the three enzymes eliminates excessive active oxygen radicals in plants and relieves the damage to plant cells due to stress, playing an important role in plants' response to drought stress (Bowler *et al*., 1992).

In this stress experiment, the activities of POD, SOD and CAT in *H. davidii* seedlings were generally increased first and then decreased with the prolongation of drought and the increase of stress degree. The results indicated that at the early stage of drought stress, under low concentration stress, the plant protective enzyme system responded, and the activities of several antioxidant enzymes were increased together to remove excessive free radicals in *H. davidii* and slow down the rate of membrane system peroxidation, thus enabling plant to resist drought stress. However, as the concentration and duration of drought stress exceeded the tolerance range of plant, it caused certain damage to the protective enzyme system, and the enzyme activity decreased. This result was similar to the findings of *Fagopyrum tataricum* under PEG stress (Huang *et al*., 2021).

Effect of PEG-6000 drought stress on chlorophyll of *Hemiptelea davidii* Leaves: In this experiment, the SPAD value of chlorophyll in leaves of *H. davidii* was decreased with the prolongation of PEG stress and the increase of stress degree. This result was consistent with previous studies on *Salvia nemorosa* under drought stress (Bayat & Moghadam, 2019). Our results indicated that drought stress inhibited chlorophyll synthesis and consequently photosynthesis in *H. davidii* seedlings, which in turn caused certain adverse effects on the growth of *H. davidii* seedlings.

Analysis of DEG under PEG-6000 drought stress: The response of plants to drought stress is a very complex process. When plants are subjected to drought stress, cells will detect and transmit drought signals, regulate gene expression and produce new protein, which will lead to changes in plant physiology and metabolism (Salvi *et al*., 2021). In this study, through DEG analysis among *H. davidii* samples under different treatments, 620 and 3803 differential expressions were obtained among w1_w2 and w1_w3 samples, respectively, indicating that with the deepening of PEG simulation of drought stress, more gene regulation and expression were involved.

GO and KEGG analysis of DEGs under PEG-6000 drought stress: Drought stress can affect the photoreaction system of plants (Vu & Allen, 2009), it has been found that after a long period of drought stress, in order to improve the utilization efficiency of photosynthetic light energy and generate more energy synthesis compounds to improve the cell permeability, a large number of genes related to plant photosynthesis are expressed (Farooq *et al*., 2009). In this

study, GO enrichment analysis of DEGs in *H. davidii* was performed, and it was found that with the prolongation of stress, more differentially expressed genes related to membrane components, photosystem, and chloroplast thylakoid cavity were enriched. Besides, it was found that a large number of DEGs among w1_w3 samples were enriched in the photosynthesis pathways, involving a large number of genes downregulated in the photosynthesis pathways such as photosystem II and photosystem I, suggesting that long-term drought stress could damage plant cell membrane and exert a certain inhibitory effect on photosynthesis.

Plants under drought stress produce a large number of transduction signals, and the catabolism of sucrose and starch in plants is enhanced. The decomposed sugars not only provide energy support for plant growth and development, but also increase the content of soluble sugar in plant cells and cellular osmotic potential to resist the hazards of drought stress (Tripathy *et al*., 2019). In this study, KEGG pathway enrichment analysis of DEGs in *H. davidii* under drought stress showed that in w1_w2 and w1_w3, DEGs were abundantly enriched in sugar metabolism pathways such as starch and sucrose metabolism, and carotenoid biosynthesis, which accelerated the decomposition and transformation process of some sugars in leaves of *H. davidii*. It could be speculated that these sugar metabolism pathways might play an important role in the molecular mechanism of PEG drought stress in *H. davidii*. In ZmBES1/BZR1-5, which has no monomeric transcriptional activity, forms a homodimer through the β-amylase (BAM) domain, which can positively regulates salt and drought tolerance by binding to E-box to induce the expression of downstream stress-related genes, and also positively regulates kernel size (Sun *et al*., 2021; Feng *et al*., 2022). In addition, it has been found that phytohormones may play a more significant role in plant adaptation to environmental changes such as drought (Peleg & Blumwald, 2011). It was found that many DEGs were enriched in plant hormone signal transduction pathways between the two groups of *H. davidii* samples. PEG stress upregulated or downregulated the expression of genes in plant hormone signal transduction pathways such as ABA and GA, involving the regulation of many transcription factor families and protein synthesis, activating ABA signal transduction, and affecting the degradation process of growth inhibitory proteins in gibberellin signal pathway, thus enhancing the resistance of *H. davidii* to drought stress. Many researchers have made plants more adaptable to drought stress by overexpressing ABA receptor protein genes. For example, overexpression of poplar *PtPYRL1* and *PtPYRL5* (Yu *et al*., 2017) and wheat *TaPYL4* (Mega *et al*., 2019) have significantly improved the drought resistance of transgenic plants.

Response of some transcription factors to PEG-6000 drought stress: Transcription factors play an important role in the regulation of environmental stress-inducible genes. Many transcription factor families such as MYB, AP2/EREBP, bHLH, NAC, and WRKY have been found to be associated with abiotic stress in plants (Wang *et al*., 2016). In the present study, many transcription factor families were involved, and the genes in these transcription factor families may have a close role in resistance to PEG drought stress. Some studies have found that the BHLH family plays an important role in abiotic stress responses and *AtbHLH112* may be involved in the regulation of salt and drought stress, and may be involved in the regulation of ABA (Min *et al*., 2015). In this study, some transcription factors in the bHLH family were involved in the regulation of abscisic acid and salicylic acid signaling transduction pathways. In addition, some genes in the bZIP transcription factor family were also involved in the plant hormone regulation pathway. It was also found that the C2H2 family involved 128 transcription factor genes, some of which were related to the functions of specific sequence DNA binding, cellular process and binding activity. Plants are subjected to drought stress and produce ethylene, which can utilize the plant's feelings and adaptation in adversity to initiate related metabolism and assist the plant's resistance to drought stress (Morgan & Drew, 1997). In the present study, transcription factors related to ethylene response were found in the AP2/ERF-ERF family. In addition, the gene binding to DNA, the activity of transcription factors, and transcriptional regulation in this family were all related. Through the joint action of these family transcription factors, the stress resistance of *H. davidii* can be enhanced to a certain extent, and the *H. davidii* can be synergistically helped to resist drought stress. Since *H. davidii* is not a model plant, its molecular mechanisms for responding to stress have not been investigated, and there is a dearth of genomic data in accessible public databases. Our findings could be used in conjunction with the complete chloroplast genome of *H. davidii* (Liu *et al*., 2019) to investigate the molecular mechanism of drought stress adaptation and serve as a foundation for *H. davidii* ' ensuing resistance breeding efforts.

Conclusion

This study explored the drought tolerance of *H. davidii* by analyzing the dynamic changes of physiological indexes with time under different degrees of drought stress. Under a certain stress duration and stress intensity of concentration, *H. davidii* has a certain drought resistance. Under PEG stress, *H. davidii* leaves lose water, wilted and drooped. *H. davidii* can resist the harm caused by drought stress by accumulating osmoregulation substances and improving the activities of some antioxidant enzymes. However, high concentration and long-term stress will destroy its own defense system and disorder, thus weakening its resistance to drought. Transcriptome analysis revealed that a large number of differentially expressed genes in *H. davidii* were involved in plant hormone regulation, antioxidant reaction and photosynthesis under high concentration (30%) PEG stress and played a regulatory role of their genes to enable *H. davidii* effectively to cope with adversity stress.

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