

Exploration of key genes and pathways in response to submergence stress in red clover (*Trifolium pratense* L.) by WGCNA

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Abstract

Background

Submergence stress is a prevalent abiotic stress affecting plant growth and development and can restrict plant cultivation in areas prone to flooding. Research on plant submergence stress tolerance has been essential in managing plant production under excessive rainfall. Red clover (*Trifolium pratense* L.), a high-quality legume forage, exhibits low tolerance to submergence, and long-term submergence can lead to root rot and death.

Results

This study assessed the microstructure, physiological indicators, and the key genes and metabolic pathways under submergence stress in the root system of red clover HL(Hong Long) and ZY(Zi You) varieties under submergence stress at 0 h, 8 h, 24 h, 3 d, and 5 d. Based on 7740 transcripts identified in the leaves at 0 h, 8 h, and 24 h submergence stress, Weighted Gene Co-expression Network Analysis (WGCNA) was performed on the differentially expressed genes (DEGs) at 8 h and 24 h. Functional annotation of the DEGs in the four key modules was obtained. Based on the results, the red clover root system exhibited epidermal cell rupture, enlargement and rupture of cortical thin-walled cells, thickening of the mid-column, and a significant increase in the number of air cavities and air cavity area of aeration tissue with the prolongation of submergence stress. The malondialdehyde content, relative conductivity, peroxidase, and superoxide dismutase initially increased and decreased as submergence stress duration increased. Four specific modules (cyan, purple, light cyan, and ivory) closely correlated with each stress were identified by WGCNA. The 14 obtained Hub genes were functionally annotated, among which six genes, including gene51878, gene11315, and gene11848, were involved in glyoxylate and dicarboxylic acid metabolism, carbon fixation in photosynthetic organisms, carbon metabolism, biosynthesis of pantothenic acid and CoA, flavonoid biosynthesis.

Conclusion

In this study, the molecular response mechanisms of red clover to submergence stress were elucidated, and the core genes and metabolic pathways in response to submergence stress were obtained, providing a valuable data resource at the physiological and molecular levels for subsequent studies of submergence stress tolerance in plants.

Introduction

Water availability is an essential environmental variable for plant growth and development and is an important component of plant photosynthesis and organic matter production. According to the Intergovernmental Panel on Climate Change (IPCC), annual rainfall will significantly increase at high altitudes and in the equatorial Pacific at the end of the 21st century, and extreme rainfall events will be more frequent in low-altitude continental regions and the wet tropics ^[1]. Such regular extreme rainfall events lead to water stagnation on the soil surface, resulting in a very low soil oxygen content ^[2]. Rainfall and water table depth affect soil hydrology and can lead to plant physiological adaptations significantly affecting anatomical features such as root depth and root morphology, as well as physiological and metabolic processes ^[3], and even cause plant yield reduction and mortality, thus seriously affecting regional ecological and economic development.

Long-term submergence stress in plants can lead to decreased plant root vigor and decreased growth indicators such as root length, root biomass, and plant height ^[4]. Submergence stress in plants can also lead to an imbalance in the production and scavenging system of reactive oxygen species, resulting in cell membrane lipid peroxidation damage ^[5]. Moreover, PS II reaction centers are damaged when plants are subjected to submergence stress, resulting in reduced photosynthetic activity and a decrease in chlorophyll content ^[6, 7]. Although water is essential for plant growth, submergence stress, by disrupting tissue oxygenation, can severely impact normal plant growth and development ^[8] and, thus, is a major constraint on the quality and yield of plants.

The plant root system is the main organ that absorbs water and mineral nutrients to support plant growth, and it is the tissue that initially senses soil-related stress. Submergence stress can quickly lead to hypoxia in the plant root system and inhibit its growth. If the plant is completely submerged, the hypoxic stress is further intensified, which can cause serious damage and even lead to plant death. Plant growth and development are determined by a combination of genetic factors and external factors, such as biotic and abiotic stresses^[9]. During their long-term evolutionary adaptation, plants have gradually evolved various morphological structures and molecular mechanisms to adapt to adverse environmental conditions^[10, 11]. Flood-tolerant plant varieties play an important role in managing extreme precipitation phenomena, reducing the cost of large-scale production and operation, expanding planting area, reducing yield losses, and improving the utilization of flooded land. Therefore, research on plant submergence stress tolerance mechanisms is important in breeding cultivars that are less affected by soil flooding.

Transcriptome sequencing of multiple samples has been widely implemented in life science research, and mining biologically significant information from the extensive transcriptome data generated has become the focus of current transcriptome analyses. Weighted gene co-expression network analysis (WGCNA)^[12, 13] is among the most effective analytical approaches for the identification of co-expression networks. It is commonly implemented for the identification of gene expression correlation modules and to predict gene regulatory relationships among genes, and thus identify biologically significant target modules and target genes^[14–16]. More recently, WGCNA approaches have also been applied in a wide range of agricultural-related research fields, such as genetic marker screening for important traits in plants and animals, plant developmental biology, reproductive biology, and pathology.

Red clover (*Trifolium pratense* L.) is a perennial cool-season herbaceous legume. It is one of the most important forage legume cultivated worldwide, including the United Kingdom, the United States, New Zealand and China. Red clover has high nutritional and medicinal value and is rich in flavonoids, proteins, amino acids, and other components^[17, 18]. It is characterized by good palatability, easy digestibility, fast regeneration, and a high biomass yield. It is rich in flavonoids and^[19] and is an important raw material for extracting flavonoids used in women's health products and medicinal products^[20]. It can be used as a green manure plant and companion plant^[21] and also as a forage plant with significantly higher utilization of protein in silage than alfalfa (*Medicago sativa*)^[22]. It prefers warm and humid climates, has poor drought tolerance but a significant tolerance to high soil moisture conditions, and is suitable for growing in regions with abundant annual precipitation and neutral alkaline soils. However, red clover does not tolerate submergence, which causes rotting and eventually plant death. Nevertheless, large differences in submergence resistance have been identified in clover cultivars.

Extensive studies have been undertaken in recent years on plant (mainly *Arabidopsis* and rice) submergence-associated stresses, such as submergence, waterlogging, hypoxia, and anoxia, to identify the underlying molecular response mechanisms of plant tolerance to submergence, including oxygen perception and signaling. In the present study, we investigated the responses of red clover seedlings under total submersion and analyzed the root morphology, microstructure, and physiological response pathways. We then conducted a WGCNA analysis based on the obtained transcriptome data, aiming to elucidate the red clover response mechanisms to submergence stress in a multidimensional manner and to uncover the core genes and the key metabolic pathways involved in submergence stress adaptation and tolerance.

Materials and Methods

Plant Growth, Submergence Stress Application, and Sample Collection

In this experiment, the flood-tolerant variety HL (Hong Long) and sensitive variety ZY (Zi You) were obtained from Beijing Best Grass Industry. The experiment was conducted at Southwest University in 2021–2022. Seeds from both varieties were sterilized with 10% H₂O₂ for 10 min during the pre-emergence period. Afterward, they were rinsed five times with sterile water to disinfect them. Subsequently, the seeds were placed in Petri dishes on a moist double layer of filter paper and incubated in a germination chamber at a constant temperature of 24°C for 4–7 days. When the cotyledons were fully expanded, seedlings with vigorous and uniform growth were selected and transplanted into pots (15.0 cm in diameter and 13.5 cm in height) containing a volume

ratio of nutrient soil: vermiculite: perlite of 3:1:1. Fifteen seedlings were transplanted in each pot. The plants were placed in an illuminated plant growth chamber at 22/15°C (day/night), 80% relative humidity, a 10,000 lux light setting, and a 16/8 h photoperiod (day/night) and watered every two days. Submergence stress was applied when the leaves were about 2 cm long and V-shaped white spots appeared on the leaf surface.

After the submergence stress treatment initiation, the leaves of HL and ZY clover seedlings were cut at 0 (CK), 8, and 24 h of stress treatment (three biological replicates at each time point). They were then immediately snap-frozen in liquid nitrogen and stored in a freezer at -80°C for subsequent transcriptome sequencing analysis. The roots were also sampled under the same submergence stress conditions at 0 (CK), 8 h, 24 h, 3 d, and 5 d and stored in a refrigerator at 4°C for microstructure and physiological index analysis.

Histological Analyses

Roots sampled at each time point of submergence stress were scanned using a Microtek ScanMaker i800 plus root scanner. The obtained images were processed with the WinRHIZO software to obtain data on phenotypic traits such as root length, diameter, surface area, and number of root tips. Paraffin sections of red clover roots under submergence stress treatment were prepared with the following steps: Extraction and fixation; dehydration and clearing of the tissue; wax immersion and embedding of the tissue; sectioning, spreading; dewaxing rehydration; and staining. The tissue sections were microscopically scanned and imaged using a PANNORAMIC panoramic section scanner (3DHISTECH, Hungary). Subsequently, observations were made at a magnification range from 1-400x using CaseViewer 2.4 software. Target areas of selected tissues were imaged at 100x to observe the structural changes in the root system under different submergence stresses of each variety.

Measurement of Physiological Indices

Physiological indices of the HL and ZY clover roots were determined at each time point of submergence stress. The electrolyte leakage method was used to measure the relative conductivity of the root system. The root malondialdehyde (MDA) content was determined using the thiobarbituric acid reactive substances (TBARS) assay^[23]. Antioxidant enzyme activities (peroxidase, POD; superoxide dismutase, SOD) were quantitatively determined using an ELISA-based protocol (TECAN, Tech Spark 10M, Zurich, Switzerland)^[24]. Three biological replicates were assessed per treatment.

Differentially Expressed Gene (DEG) Screening and Weighted Gene Co-expression Network Analysis (WGCNA)

In order to obtain transcript information under submergence stress for the red clover HL and ZY varieties, total RNA was extracted from seedling leaves of both varieties at 0, 8, and 24 h under submergence stress using the Trizol kit (Invitrogen, USA). The extracted RNA concentration and purity were assessed using Nanodrop 2000 (Thermo Scientific, USA). The RNA Nano 6000 assay kit for the Agilent Bioanalyzer 2100 system (Agilent Technologies, USA) was used to assess the RNA integrity to ensure that high RNA-quality samples were used for transcriptome sequencing. Subsequently, cDNA sequencing libraries were constructed from the samples that passed the quality control. The library concentration was accurately quantified using q-PCR. The cDNA libraries were pooled based on the target sequencing depth and coverage and then sequenced using the Illumina high-throughput sequencing platform based on the sequencing by synthesis (SBS) technology. Before data analysis, the raw data were filtered, and high-quality clean data were obtained after strict quality control, such as removing the adapter sequences from the reads and removing low-quality reads. The obtained high-quality clean data were aligned and mapped to the reference clover genome based on the HISAT2^[25] alignment software. The reference genome is available for download at: ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/583/005/GCA_000583005.2_Tp1.0/GCA_000583005.2_Tp1.0_genomic.fna.gz. After mapping and assembly, the reads were quantitatively analyzed using String Tie. Differential expression analysis between treatment groups was performed using DESeq2. Fold Change ≥ 1.5 and P -value < 0.01 were used as the screening criteria to compare the gene expression levels in the leaves of seedlings of the two varieties at different periods of submergence stress and to identify DEGs between the comparison groups.

Transcripts with an absolute value of FPKM > 1, obtained from the HL and ZY clover varieties under submergence stress treatments at 0 h, 8, and 24 h, were screened for differential expression. Co-expression networks of differentially expressed genes were constructed using the R software and the WGCNA package for gene expression profile matrices (Langfelder et al., 2008). Firstly, the gene network was assumed to obey a scale-free distribution, and the two-by-two correlation coefficients between genes were calculated. The Pearson correlation coefficient for each pair of gene i and gene j was S_{ij} , and the gene co-expression correlation matrix $S=[S_{ij}]$ was constructed using $S_{ij}=|\text{cor}(i,j)|$, where S denotes the similarity matrix. Then, the threshold value of the correlation coefficient between the specified genes was determined using the power exponential adjacency function $a_{ij} = \text{power}(S_{ij}, \beta) = |S_{ij}|^\beta$, where a_{ij} is used as a measure of the relationship between gene i and gene j. According to the principle of scale-free networks, the parameter weighting factor β of the adjacency function was defined. Subsequently, a topological overlap dissimilarity measure (dissTOM) was performed. The matrix S was first converted into an adjacency matrix $A=[a_{ij}]$, and subsequently, the adjacency matrix was converted into a topological overlap matrix (TOM), $\text{TOM} = \text{direct correlation} + \text{indirect correlation}$. In order to eliminate the error caused by pseudo-association, $\text{diss TOM} = 1 - \text{TOM}$ was used to calculate the dissimilarity between genes and obtain the dissimilarity matrix. Finally, the hierarchical clustering tree between each two genes was constructed based on the dissimilarity coefficients, and the resulting clustering tree was cut using Dynamic tree cut (DTC) to identify gene co-expression modules. To merge modules with similar expression and identify a greater number of significant modules, the module similarity threshold was set to 0.25, and the minimum number of genes per gene network module was set to 30. This process merged genes with similar expression patterns on the same branch, with each branch representing a co-expression module and each color corresponding to genes on the clustering tree belonging to the same module. Each module eigengene (ME) and the correlation of the eigenvectors of the resulting modules with the treatments were calculated. Modules with a correlation greater than 0.8 were selected as the modules that were significantly correlated with each treatment. After identifying the significantly correlated modules, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) annotations and enrichment analyses were performed for the genes in the module, and finally, the co-expression network was visualized using Cytoscape (version 3.9.0).

qRT-PCR Validation

Eight co-expressed DEGs were randomly selected, as well as Hub genes identified from WGCNA. To validate the transcriptome sequencing results, quantitative real-time PCR (qRT-PCR) analysis was performed on the selected genes using the *GAPDH* gene as the internal reference gene (Zaffagnini et al., 2013). The primers used are listed in (Table S1). The PrimeScript™ RT reagent Kit with the gDNA Eraser kit (Takara, Dalian, China) was used to reverse transcribe RNA into cDNA. qRT-PCR was performed with the TB Green Premix Ex TaqTM II kit (Takara, Dalian, China), and the reaction system is shown in (Table S2). Three technical replicates were performed for each target gene. PCR amplification was performed using a three-step method: pre-denaturation at 95°C for 30 s; 40 cycles of 95°C for 15 s, 60°C for 1 min; melt curve analysis at 95°C for 15 s, 60°C for 1 min, 95°C for 15 s. Finally, the relative expression of genes was calculated using the $2^{-\Delta\Delta C_t}$ method [26].

Results

Clover morphological features and changes under submergence Stress

Red clover plants became elongated, and both the aboveground parts and root system grew after 8 and 24 h of submergence stress (Fig. 1a). At 3 d of submergence stress, leaves of both HL and ZY clover varieties showed signs of decay, the tips of their root system gradually turned black, and the length of the root system was gradually reduced. At 5 d of submergence, the aboveground tissues of ZY were severely decayed, and the number of leaves was reduced due to leaf abscission. At 24 h of submergence, the root length of the HL and ZY varieties reached their maximum values of 63.10 mm and 53.55 mm, respectively. HL had significantly longer root than ZY and showed a highly significant difference at 3 and 5 d (Fig. 1b). Both HL and ZY exhibited a trend of an initially increasing and then decreasing root surface area, with maximum values of 7.12 mm² and 7.23 mm² at 24 h of submergence stress. However, no significant differences were observed between the varieties (Fig. 1c). The HL and ZY root diameters reached a maximum value of 0.36 mm and 0.44 mm at 24 h of submergence stress. ZY showed the most significant differences at 24 h and 3 d, having a 23.06% and 13.43% greater root diameter compared to HL (Fig. 1d). Moreover, HL had a higher number of root tips compared to ZY at all time points of submergence stress, with significant

differences between varieties at 24 h and 3 d of submergence stress (Fig. 1e). Taken together, these results suggest that the different submergence symptoms expressed in HL and ZY root morphology at different time points of submergence stress may be due to the differences in submergence tolerance between the two varieties due to genotypic differences.

Microstructure of the Root System under Submergence Stress

Root anatomical properties represent the most direct manifestation of the level of root development and are intricately linked to physiological functions. In this study, it was found that submergence stress significantly impacted the microstructure of the red clover root system. Based on root transverse section observations (Fig. 2a), the increase in submergence duration resulted in an increased thickening of the mid-columns of both HL and ZY varieties. The number of conduits exhibited an increasing trend in both HL and ZY at all time points of submergence stress, showing highly significant differences at 8 h and 24 h, with 50% and 15.56% more conduits in HL than ZY (Fig. 2b). However, the conduit diameter did not significantly differ (Fig. 2c). HL and ZY were significantly thickened at both 8 and 24 h of submergence stress and reached a maximum value of 0.32 mm and 0.26 mm at 24 h. At 3 and 5 d of submergence stress, a significant reduction of the cortex thickness of ZY was observed, with the most significant change at 5 d, with the cortex thickness measured at 0.16 mm (Fig. 2d). The vascular column diameters of both HL and ZY showed a gradual increase with the increase in the duration of submergence stress, with significant differences observed at each time point. The vascular column diameter of HL was significantly longer compared to ZY at 24 h and 3 d (Fig. 2e). In conclusion, red clover may adapt to short-term submergence mainly by increasing the number of root conduits, cortex thickness, and vascular column diameter at the microstructural level.

Physiological Indices of the Root System under Submergence Stress

The relative conductivity, malondialdehyde content, peroxidase, and superoxide dismutase activities of HL and ZY roots exhibited a trend of initially increasing and then decreasing with the increase in submergence stress duration. Among them, the relative conductivity reached its maximum values at 24 h, with ZY consistently exhibiting a higher relative conductivity compared to HL (Fig. 3a), which was significantly higher at 5 d of submergence ($P < 0.01$). This indicated that submergence stress had a more significant effect on membrane permeability in ZY. Malondialdehyde content reached its maximum values at 24 h of submergence stress in HL. At the later time points, it was lower compared to ZY, indicating that the degree of membrane lipid peroxidation was differentially affected in the two varieties. It also suggested that HL was more sensitive to submergence stress in the initial submergence stage (Fig. 3b). The SOD activity in the root systems of HL and ZY did not exhibit a significant trend at all time points of submergence stress (Fig. 3c). On the other hand, the POD activity in the root systems of HL and ZY tended to increase with the increase of submergence stress duration and eventually decreased at later points in time. Specifically, at 8 h of submergence stress, the POD activity in the root systems of both HL and ZY was significantly increased, reaching its maximum value, and then showed a decreasing trend (Fig. 3d).

Identification of Differentially Expressed Genes

The raw sequencing data from 18 samples generated by Illumina's high-throughput sequencing platform were subjected to strict quality control. They yielded a total of 124.53Gb of high-quality clean data, with Q30 percentages of 93.58% and above. The clean reads of each sample were compared with the designated reference genome for sequence alignment, with the alignment coverage ranging from 77.54–83.50% (Table S3). Gene expression analysis was then performed based on the processed and aligned sequencing reads. Differentially expressed genes (DEGs) were identified based on their expression in different samples and were used for subsequent analyses.

To obtain the DEGs between the HL and ZY varieties under submergence stress at 8 and 24 h, different comparison groups were designated compared to the control: H8h vs. 0h, H24h vs. 0h, Z8h vs. 0h, Z24h vs. 0h. Differential expression analysis was performed using DESeq2, and Fold Change ≥ 1.5 and P -value < 0.01 to identify the DEGs. The screening criteria were used to determine the number of DEGs within each comparison group. The results are shown in (Fig. S1, Fig. 4a) (Table 1). The number of down-regulated genes was greater than the number of up-regulated genes in both varieties after submergence stress, and the number of differentially expressed genes increased after 24 h of submergence stress.

Table 1
Statistics of differentially expressed genes in different treatment comparison groups

Variety	Comparison groups	Total	Up	Down	Unique	Common
HL	H8h vs 0h	5,060	2,440	2,620	1036	4024
	H24h vs 0h	9,016	4,277	4,739	4992	
ZY	Z8h vs 0h	5,040	2,244	2,796	1491	3549
	Z24h vs 0h	6,570	2,922	3,648	3021	

Construction and Analysis of Weighted Gene Co-expression Networks

WGCNA implements a clustering algorithm for constructing gene co-expression networks based on differential expression data. In this study, a total of 7740 transcripts with FPKM absolute values > 1 were selected as differentially expressed between the two varieties of red clover ZY and HL under submergence stress at 0, 8, and 24 h to construct gene co-expression matrices. Co-expression networks were constructed utilizing the R software and the WGCNA package. The degree of gene co-expression in different modules was determined, and the association between gene expression modules and each treatment was explored to identify target genes and their respective networks.

Initially, a soft threshold was determined. To measure whether genes exhibited similar expression patterns and to ensure that the connections between genes obey the scale-free network distribution, the square of the correlation coefficient (R^2) was set to 0.8, above which the expression patterns are considered similar. Higher R^2 values indicate that the network approximates a scale-free distribution. In this study, $\beta = 14$ ($R^2 > 0.8$) was chosen to construct a weighted gene co-expression network (Fig. 4b). Next, module hierarchical clustering was performed. The hierarchical clustering tree among genes was constructed based on the TOM value among each gene node, and the gene co-expression modules were divided, as shown in (Fig. 4c). Each color in the figure represents genes on the corresponding clustering tree that belong to the same module, and the number of different colors represents the number of modules. 13 gene co-expression modules were eventually obtained. The number of genes in each module is shown in (Table S4). Finally, module gene-clustering was performed. The H-clust function of the R package was used to construct a module-gene clustering heat map for the 13 modules obtained above (Fig. 4d).

In general, if the correlation between a module and a sample is significantly higher than other modules, it indicates that this specific module may have the strongest association with that treatment. Based on this information, the module-sample correlation heat map was constructed, as shown in (Fig. 4e) below. Based on the results, the cyan and purple modules had very high correlations with samples H8 and H24, respectively (correlation coefficients were 0.96 and 0.99 with P -values of 0.003 and 6e-05). This suggests that genes in the cyan and purple modules may be involved in the response of the HL variety to submergence stress at 8 and 24 h. It is worth noting that the light cyan module was strongly correlated with sample H0 (correlation coefficient 0.94, P -value 0.006) and exhibited a negative correlation with other samples of HL as the submergence stress duration increased, suggesting that the genes in the light cyan module may have a negative regulatory effect on the response of HL to submergence stress. However, none of the above modules showed a significant correlation among the ZY samples. The ivory and light green modules in ZY exhibited significant correlations with Z8 and Z24, respectively (correlation coefficients of 0.92 and 0.83, P -values of 0.009 and 0.04). Similarly, these two modules did not exhibit any correlation with the HL samples. This suggests that there may be significant differences in gene expression regulation between the submergence-tolerant and sensitive varieties HL and ZY in response to submergence stress.

GO Enrichment Analysis in Key Gene Modules

The four modules, cyan, purple, light cyan, and ivory, with the highest correlation coefficients with the samples and the strongest significance, were selected for GO enrichment analysis. The 211 DEGs in the cyan module were annotated into 40 GO terms, containing 15 biological process GO terms, 14 cellular component GO terms, and 11 molecular function GO terms (Fig. S2a). The 215 DEGs in the purple module were annotated into 37 GO terms, containing 15 biological process GO terms, 15 cellular component GO terms, and 7 molecular function GO terms (Fig. S2b). The 258 DEGs in the light cyan module were annotated

into 33 GO terms, containing 12 biological process GO terms, 11 cellular component GO terms, and 10 molecular function GO terms (Fig. S2c). The 249 DEGs in the ivory module were annotated into 30 GO terms, containing 13 biological process GO terms, 12 cellular component GO terms and 5 molecular function GO terms (Fig. S2d). The genes in the four modules with the strongest correlations with the samples were enriched to varying degrees in the GO categories redox process, enzyme activities such as oxidoreductases and hydrolases, endomembrane system, cell cortex, cell wall, and terpene metabolism, suggesting the involvement of these pathways in the responses of red clover to the submergence stress. Meanwhile, the light cyan module genes, which were significantly correlated with the H0 samples that corresponded to the control, were significantly enriched in carbohydrate metabolism, fatty acid metabolism, and lipid transport. This module exhibited a negative correlation with H8 and H24 samples of submergence stress. Therefore, we hypothesized that submergence stress might affect carbohydrate metabolism, fatty acid metabolism, and lipid transport processes in red clover.

Key Gene Module Pathway Enrichment Analysis

To further explore the functions of genes in the identified modules and to gain a deeper understanding of the red clover response mechanisms to submergence stress, we used the Cluster Profiler software to identify the enriched KEGG pathways among genes in cyan, purple, light cyan, and ivory modules at $P < 0.05$ (Table S5).

There were 139 genes annotated to 52 metabolic pathways in the cyan module, among which the significantly enriched pathways were flavonoid biosynthesis, glyoxylate and dicarboxylate metabolism, fatty acid elongation, and carbon metabolism (Fig. 5a). 140 genes were annotated to 69 metabolic pathways in the purple module, with significant enrichment of pathways such as homologous recombination, alanine, aspartate and glutamate metabolism, and phenylpropanoid biosynthesis, nucleotide excision repair (Fig. 5b); 187 genes were annotated to 59 metabolic pathways in the lightcyan module, among which the significantly enriched pathways were glycosphingolipid biosynthesis, other glycan degradation, sphingolipid metabolism, glycosaminoglycan degradation, fatty acid degradation, fatty acid biosynthesis, fatty acid metabolism, starch and sucrose metabolism (Fig. 5c). Finally, 106 genes were annotated to 54 metabolic pathways in the ivory module, which were significantly enriched in the carotenoid biosynthesis, sesquiterpenoid and triterpenoid biosynthesis, pantothenate and CoA biosynthesis pathways (Fig. 5d).

The results suggest that the red clover responses to submergence stress might involve the pathways of flavonoid biosynthesis, glyoxylate and dicarboxylic acid metabolism, carbon metabolism, amino acid, and terpenoid biosynthesis. Notably, genes in the light cyan module, significantly associated with the unflooded stress H0 samples, were significantly enriched in fatty acid degradation, fatty acid metabolism, and sugar metabolism pathways. Moreover, the genes in this module exhibited a negative correlation with the samples corresponding to submergence stress. Thus, consistent with our suggestion above, submergence stress may have inhibited fatty acid metabolism and starch and sucrose biosynthesis.

Construction and Visualization of Key Gene Interaction Networks

The Cytoscape software was used to map the gene interactions in the target modules, and the nodes with weight > 0.5 in each module were selected for subsequent analysis. The degree was calculated using the Centiscape 2.2 plug-ins, and the size of the nodes was displayed according to the degree size. The Hub genes obtained from the gene network interaction analysis in the cyan module were *gene53873*, *gene51878*, *gene51609*, and *gene64012* (Fig. 6a), in the purple module were *gene48428*, *gene45795*, and *gene39564* (Fig. 6b), in the lightcyan module were *gene4283*, *gene38670*, and *gene37375* (Fig. 6c), and in the ivory module were *gene29857*, *gene11848*, *gene11315*, *gene59154* (Fig. 6d).

The functional annotation of the above 14 Hub genes is shown in (Table 2). The genes *gene11315*, *gene11848*, *gene29857*, *gene51878*, *gene45795*, *gene4283*, based on their annotation, were assigned to functions such as RNA degradation, glyoxylate and dicarboxylic acid metabolism, carbon fixation in photosynthetic organisms, carbon metabolism, pantothenic acid and CoA biosynthesis, circadian-vegetative, flavonoid biosynthesis, keratin folinic acid and wax biosynthesis, and degradation of other sugars across nine metabolic pathways. These six genes may have key functions in the response of red clover to submergence stress. We suggest that red clover may activate carbon metabolism and material translocation in the early stage of submergence stress. It may also respond to the hypoxic environment by promoting the synthesis of corresponding enzymes

and flavonoids, etc. The plants tend to senesce with the prolongation of stress supporting the enrichment of RNA degradation, which is one of the key markers of plant senescence.

Table 2 Functional annotation of Hub genes in the key modules			
Module	Gene ID	KEGG pathway	Nr annotation
ivory	gene11315	RNA degradation	hypothetical protein L195_g011316
	gene11848	Glyoxylate and dicarboxylate metabolism	ribulose biphosphate carboxylase small chain 3A
		Carbon fixation in photosynthetic organisms	nodulin 6 hypothetical protein TSUD_324920
cyan	gene29857	Carbon metabolism	protease inhibitor, partial
purple	gene51878	Pantothenate and CoA biosynthesis	cytosolic endo-beta-N-acetylglucosaminidase-like protein
light cyan	gene45795	Circadian rhythm – plant	
	gene4283	Flavonoid biosynthesis	
		Cutin, suberin, and wax biosynthesis	
		Other glycan degradation	

qRT-PCR Validation

In order to verify the validity of the transcriptome sequencing data, we performed qRT-PCR expression analysis on the eight co-expressed DEGs identified. As determined by the correlation analysis for these eight genes, the R^2 between qRT-PCR and RNA-Seq in HL was 0.8719 (Fig. 7a) and 0.7911 in ZY (Fig. 7b). This indicated that the qRT-PCR results were highly consistent with the expression levels from the RNA-seq sequencing results, indicating that the data obtained by transcriptome sequencing were highly reliable.

Further validation of the above six genes (*gene11315*, *gene11848*, *gene29857*, *gene51878*, *gene45795*, and *gene4283*) revealed that *gene29857*, *gene11848*, and *gene51878* were expressed in all samples, and the expression levels of these genes in different treatment groups were different (Fig. 7c). It further suggests that genes *gene29857*, *gene11848*, and *gene51878* may be candidates that play key roles in the regulation of red clover responses to submergence stress by influencing pantothenic acid and CoA biosynthesis, glyoxylate and dicarboxylic acid metabolism, carbon fixation in photosynthetic organisms, carbon metabolism, circadian-plant, and flavonoid biosynthetic pathways.

Discussion

The plant root system is the main organ that absorbs water and mineral nutrients to maintain normal plant growth and is also the first organ that senses soil-related stress conditions. Its growth and development are determined by genetic factors, external biotic stresses, and abiotic stresses and their interactions^[19]. When plant roots absorb water from the soil, it is first transported through cortical tissues and then upward through the xylem. Some studies have shown that the structure and properties of cortical tissues, the thickness of the inner and outer cortical cell walls, and the number and diameter of xylem vessels are altered according to the degree of stress^[27]. Submergence stress also leads to disruption of the dynamic homeostasis mechanism of reactive oxygen species in plants, resulting in metabolic disorders and electron leakage in the cells. This results in the excessive accumulation of superoxide anion radicals ($O_2^{\cdot-}$), hydroxyl radicals, hydrogen peroxide (H_2O_2), nitric oxide, and other ROS, causing damage to the plant membrane system and cellular oxidation^[28]. Among them, the synthesis of antioxidant compounds not only scavenges ROS in plants but, at the same time, synergistically increases the activity of related enzymes to improve plants' tolerance to submergence stress. Studies have shown that the increase in SOD, CAT, and APX activities in rice^[29], *Nicotiana tabacum* L.^[30], and *Triticum aestivum* L.^[31] under submergence stress plays an important role in submergence tolerance. However, as submergence stress conditions are prolonged or get more severe, the antioxidant

protection system is severely damaged, leading to the continuous accumulation of ROS. It also leads to a gradual increase in the content of malondialdehyde, a cellular membrane lipid peroxidation product, which will trigger and further exacerbate membrane lipid peroxidation, causing severe damage to cell membrane function and eventually leading to plant death.

Weighted gene co-expression network analysis (WGCNA) is an efficient clustering analysis method to identify biologically important gene co-expression modules and obtain candidate genes by correlating them with target traits^[12, 13]. In this study, based on the transcriptome sequencing results from leaves of the flood-tolerant "HL" and sensitive "ZY" red clover varieties under 0, 8, and 24 h of submergence stress, 7740 transcripts were selected for gene co-expression network construction and co-expression module identification. GO functional enrichment and KEGG functional annotation of the genes in the identified expression modules (cyan, purple, light cyan, and ivory) revealed that red clover mainly engaged the flavonoid biosynthesis, glyoxylate and dicarboxylic acid metabolism, carbon metabolism, fatty acid metabolism, circadian-vegetative, pantothenic acid and CoA biosynthesis and other metabolic pathways in response to submergence stress.

Roles of Flavonoid Biosynthetic Pathways in Submergence Stress

Flavonoids are an important polyphenolic secondary metabolite involved in plant growth and defense responses with key functions and properties such as antibacterial, antioxidant, and free radical scavenging^[32]. It has been demonstrated that plants can respond and adapt to biotic and abiotic stresses by the accumulation of compounds such as phenols and flavonoids^[33, 34]. A previous study^[35] on phenolic and other secondary metabolite accumulation under water stress in *Chrysanthemum morifolium* L. revealed significant differences in DPPH antioxidant activity, and the accumulation of phenols, flavonoids, and anthocyanins between control and water-deprived plants. The content of compounds such as quercetin and apigenin increased with increasing water stress as measured by high-performance liquid chromatography analysis. It has also been suggested that polyphenolic compound accumulation under water stress may be related to stress intensity, the plant growth stage, or plant species and that water submergence stress may promote the biosynthesis of compounds such as flavonoids^[36]. Studies in *Vitis vinifera* L.^[37], *Triticum aestivum* L.^[38], and *Reaumuria soongorica* L.^[39] revealed a higher accumulation of these compounds under submergence stress. The results of this study showed that genes in both the cyan and ivory modules (significantly correlated with samples H8 and Z8, respectively) were enriched in the flavonoid biosynthetic pathway, which was significantly enriched in the cyan module ($P < 0.05$), with the Hub gene *gene 51878* in this module was also annotated in this pathway. However, the ivory module did not exhibit a significant enrichment in the flavonoid biosynthetic pathway. In response to submergence stress, flavonoid biosynthesis in the flood-tolerant variety HL was altered more significantly than in the sensitive variety ZY, suggesting genotypic differences in the flavonoid biosynthesis pathway in response to submergence stress in red clover^[40] also found that flavonoid content was affected by water deficit and varietal interaction effects, which is consistent with the results of the present study.

Role of Carbon Metabolic Pathways in Submergence Stress

Carbon metabolism plays a decisive role in biological growth and amino acid biosynthesis^[41, 42]. Iwmer^[43] found that during induced and targeted metabolic modifications in plants, cellular equilibrium is disrupted, carbon metabolism is inhibited, leading to excess carbon sources, and cells synthesize amino acids in large quantities or initiate other non-related metabolic pathways, resulting in metabolic inefficiencies Yalage et al.^[44] investigated the combined effect of carbon concentration and incubation temperature on the biosynthesis of VOCs was investigated using a Box-Behnken experimental design. In this study, the carbon metabolism pathway was significantly enriched in the cyan module based on KEGG enrichment analysis. On the other hand, the biosynthetic pathways of amino acids such as alanine, aspartate, and glutamate were enriched in the purple module. Therefore, we speculate that submergence stress may have disturbed the metabolic balance in HL, causing carbon accumulation and excess carbon sources in the pre-submergence period, thus inducing the synthesis of organic compounds such as amino acids to maintain the energy supply required for red clover growth under submergence stress.

Role of the Glyoxylate Pathway under Submergence Stress

The glyoxylate pathway, also known as the glyoxylate cycle, is a process that is only present in plants and microorganisms functioning in lipid-to-sugar conversion. It plays an important role in plant growth and development and resistance to stress

conditions and has been relatively well-studied in rice ^[45]. Among the three catalase isozymes isolated from tobacco (*Nicotiana tabacum*), CAT1 is mainly responsible for scavenging H₂O₂ produced by photorespiration, CAT2 scavenges H₂O₂ produced by oxidative stress, and CAT3 mainly scavenges H₂O₂ produced by the glyoxylate cycle ^[46]. In the present study, the glyoxylate and dicarboxylic acid metabolism pathways were enriched among the genes of both cyan and purple modules, and they were significantly enriched in the cyan module, suggesting that these pathways play an important role in the pre-submergence stress of HL. The light cyan module genes were significantly enriched in fatty acid degradation, fatty acid biosynthesis, fatty acid metabolism, starch, and sucrose metabolism. Notably, the light cyan module was strongly correlated with sample H0. We speculate that submergence stress may have induced the expression of genes such as *gene11848* in the glyoxylate and dicarboxylic acid metabolism pathways in red clover, thus enhancing the conversion of lipids to sugars to adapt to short-term submergence stress.

Fatty acid biosynthesis is one of the fundamental metabolic processes in plants, and regulating their metabolism can enhance the organism's resilience ^[47, 48]. Unsaturated fatty acids are components essential for plant membrane function. It has been demonstrated that specific ratios of saturated and unsaturated fatty acids in membrane lipids are required for photosynthetic thermal stability and adaptation to high temperatures and that inter-organ transfer of fatty acids is involved in mediating temperature-induced membrane changes and adaptation to high temperatures ^[49, 50]. When the relevant enzymes that catalyze the biosynthesis of unsaturated fatty acids are mutated, the amount of unsaturated fatty acids in plants is reduced, leading to reduced cold resistance ^[51]. The fatty acid biosynthesis and degradation pathways were significantly enriched in the light cyan module in this study, suggesting that fatty acid metabolism may be involved in red clover responses to submergence stress.

Conclusion

Based on transcriptome sequencing, we comprehensively assessed the transcriptome of the red clover varieties ZY and HL under submergence stress at 0, 8, and 24 h, with a total of 7740 DEGs identified between the genotypes and submergence stress time points. Four specific modules tightly associated with submergence stress adaptation of each variety were identified using weighted gene co-expression network analysis (cyan, purple, light cyan, and ivory modules). The 14 obtained Hub genes were functionally annotated, of which six genes, including *gene51878*, *gene11315*, and *gene11848*, were annotated to RNA degradation, glyoxylate, and dicarboxylic acid metabolism, carbon fixation in photosynthetic organisms, carbon metabolism, biosynthesis of pantothenic acid and CoA, circadian rhythm - plants, flavonoid biosynthesis, terpenoid biosynthesis, and degradation pathways of other sugars, providing significant insights on the core genes involved in red clover responses to submergence stress. In this study, the molecular response mechanism of red clover adaptation to submergence stress was revealed for the first time, and the core genes and metabolic pathways were identified, providing a valuable data resource for subsequent studies of submergence stress tolerance in red clover and other plants.

Declarations

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Authors' contributions

BZ and PPS conceived and designed the project and the strategy. PPS, LB and WWL contributed to plant samples collection. PPS, XLZ and YLF performed experiments. PPS analyzed data and wrote the manuscript. JHW revised the manuscript. All authors approved the submitted version.

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Data availability

All raw sequence reads are available from the NCBI under the Project CRA009651. All other relevant data were included in the paper and Supplementary Materials.

Ethics approval and consent to participate

We all declare that manuscript reporting studies do not involve any human participants, human data, or human tissue. So, it is not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Figures

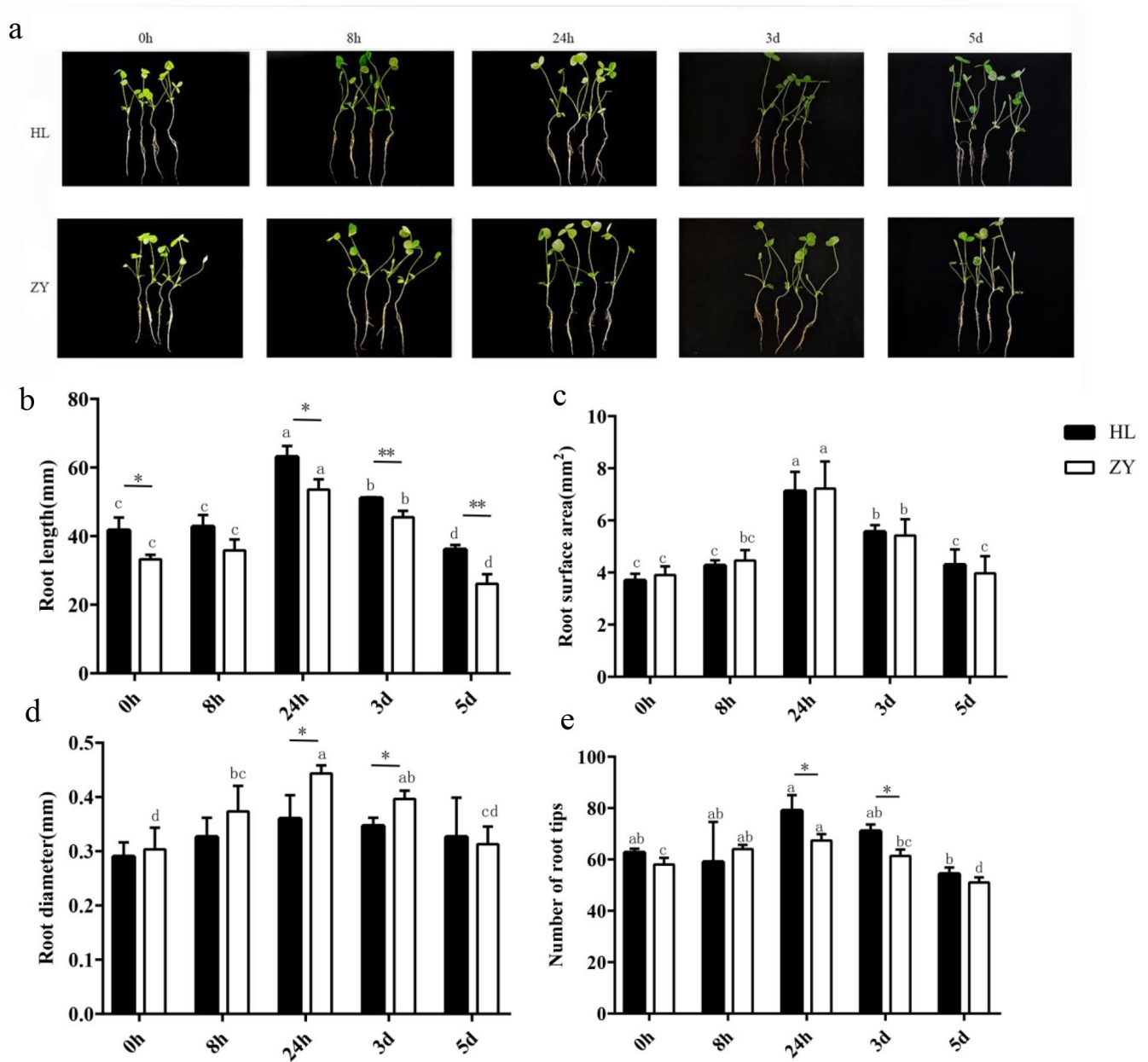


Figure 1

Effects of submergence stress on morphological indicators of red clover. (a) Morphological changes. (b) root length. (c) root surface area. (d) root diameter. (e) number of root tips.

Different lowercase letters indicate significant differences ($P < 0.05$) between different submergence stress treatment time points of the same variety; * indicates significant differences ($P < 0.05$) between different varieties of the same submergence stress treatment time points, ** indicates highly significant differences ($P < 0.01$) between different varieties of the same submergence stress treatment time points, the same below.

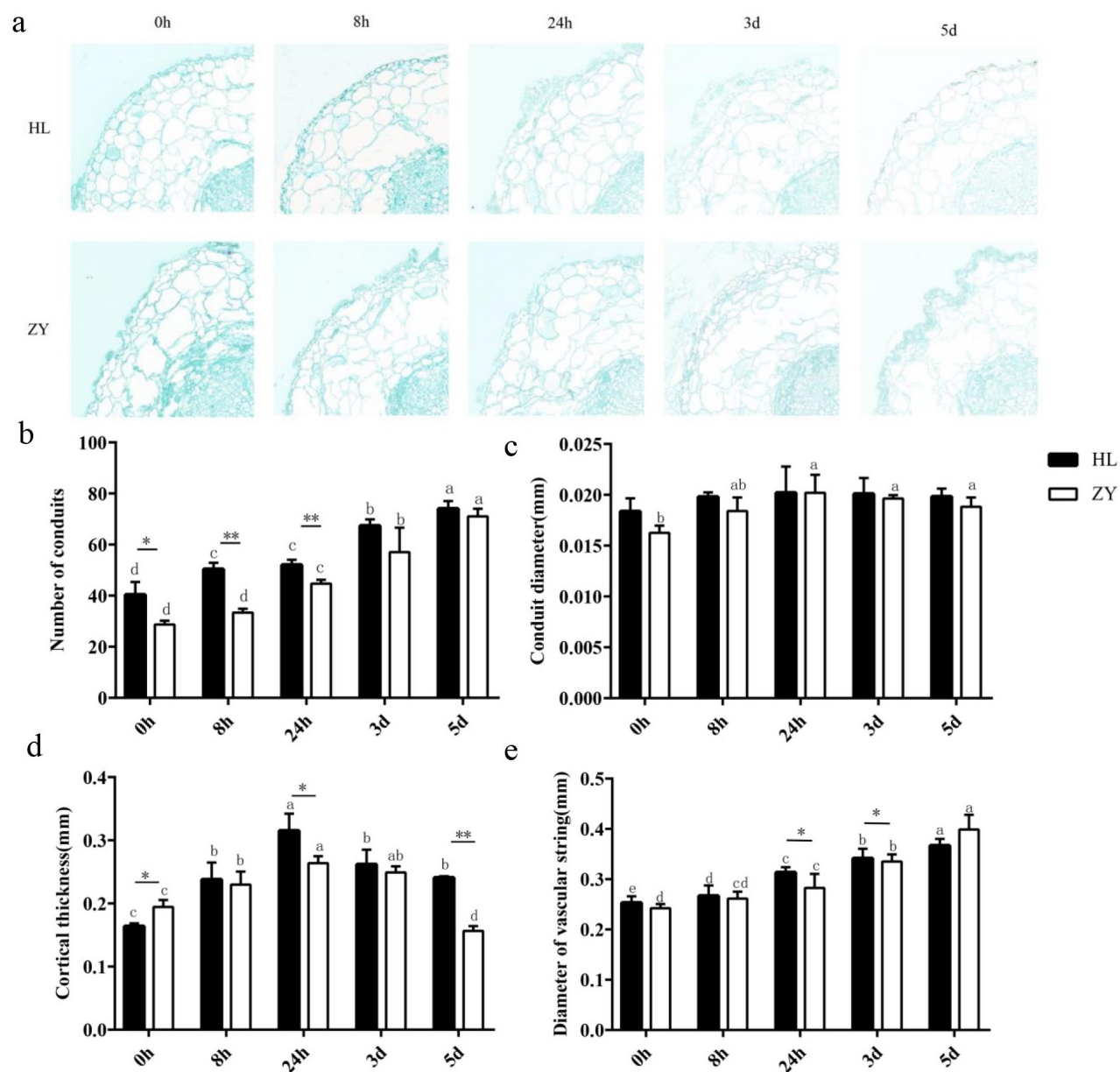


Figure 2

Effects of submergence stress on the microstructure of red clover root systems. (a) Microstructure of the root system. (b) number of conduits. (c) conduit diameter. (d) cortical thickness. (e) diameter of vascular string.

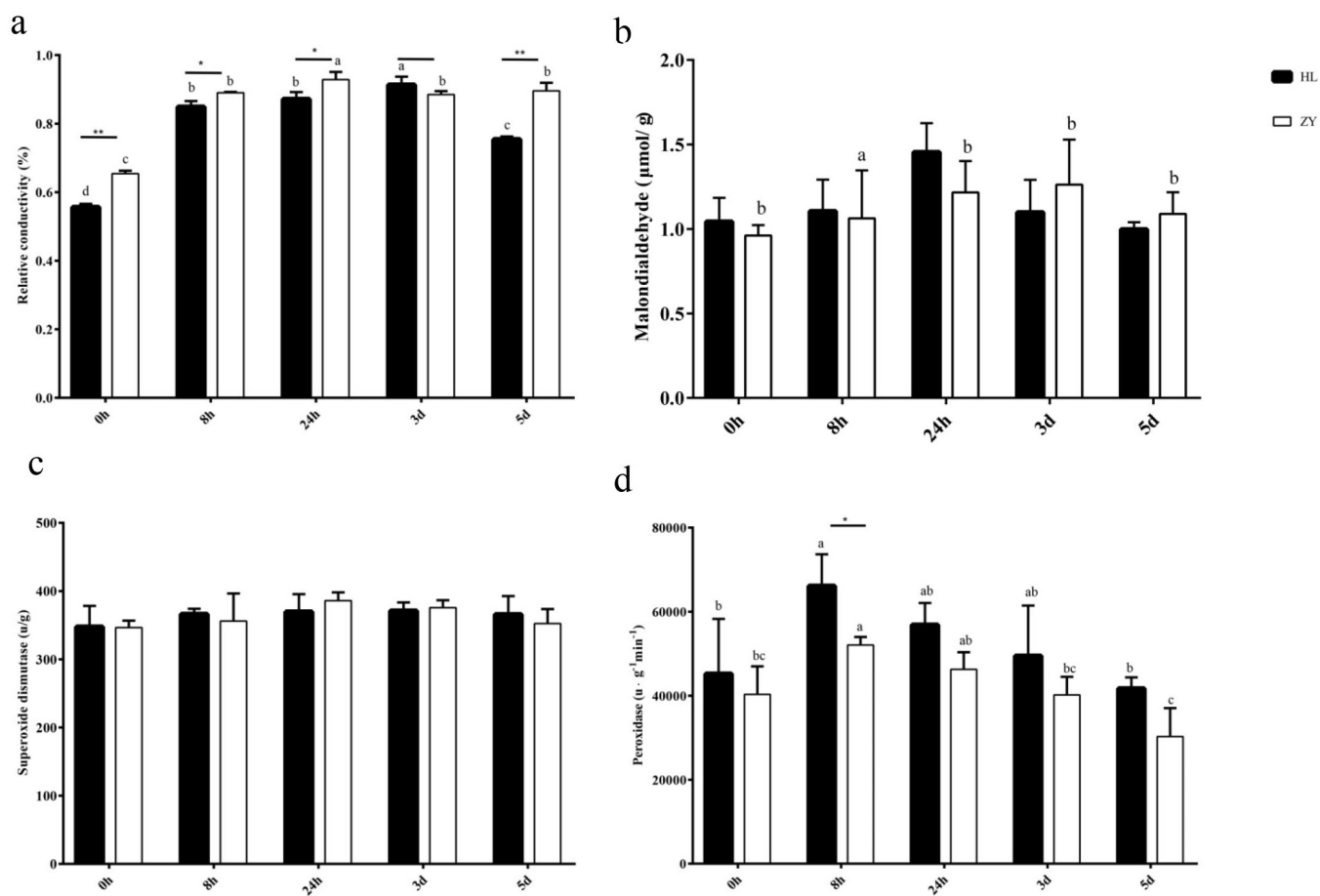


Figure 3

Effect of submergence stress on (a) relative conductivity, (b) malondialdehyde, (c) superoxide dismutase and (d) peroxidase of red clover roots.

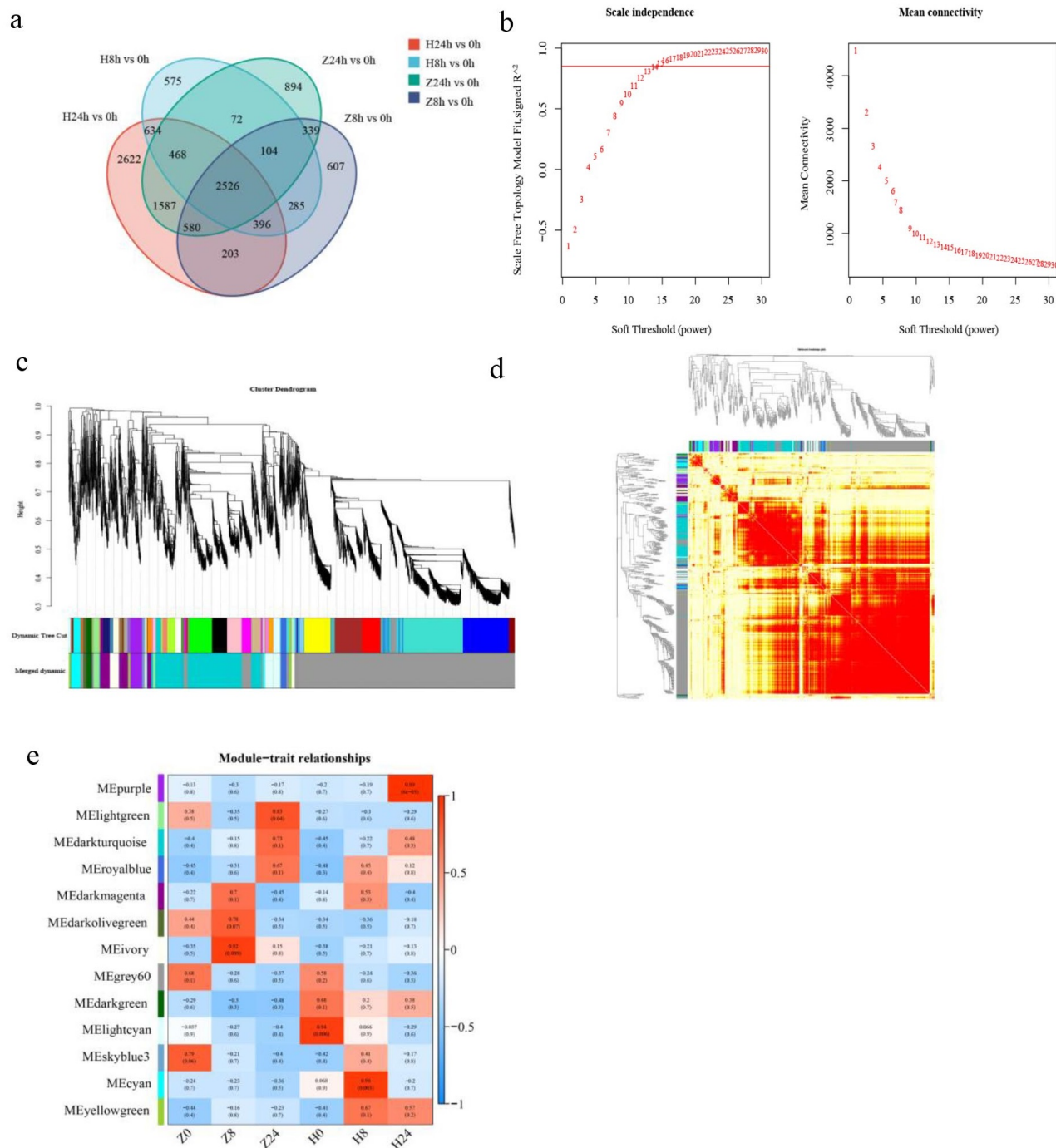


Figure 4

(a) Venn diagram of differentially expressed genes in each treatment group under submergence stress. (b) Soft threshold (power) selection diagram. (c) Module hierarchical clustering tree diagram. (d) Module gene clustering heat map. (e) Heat map of correlation between samples and modules.

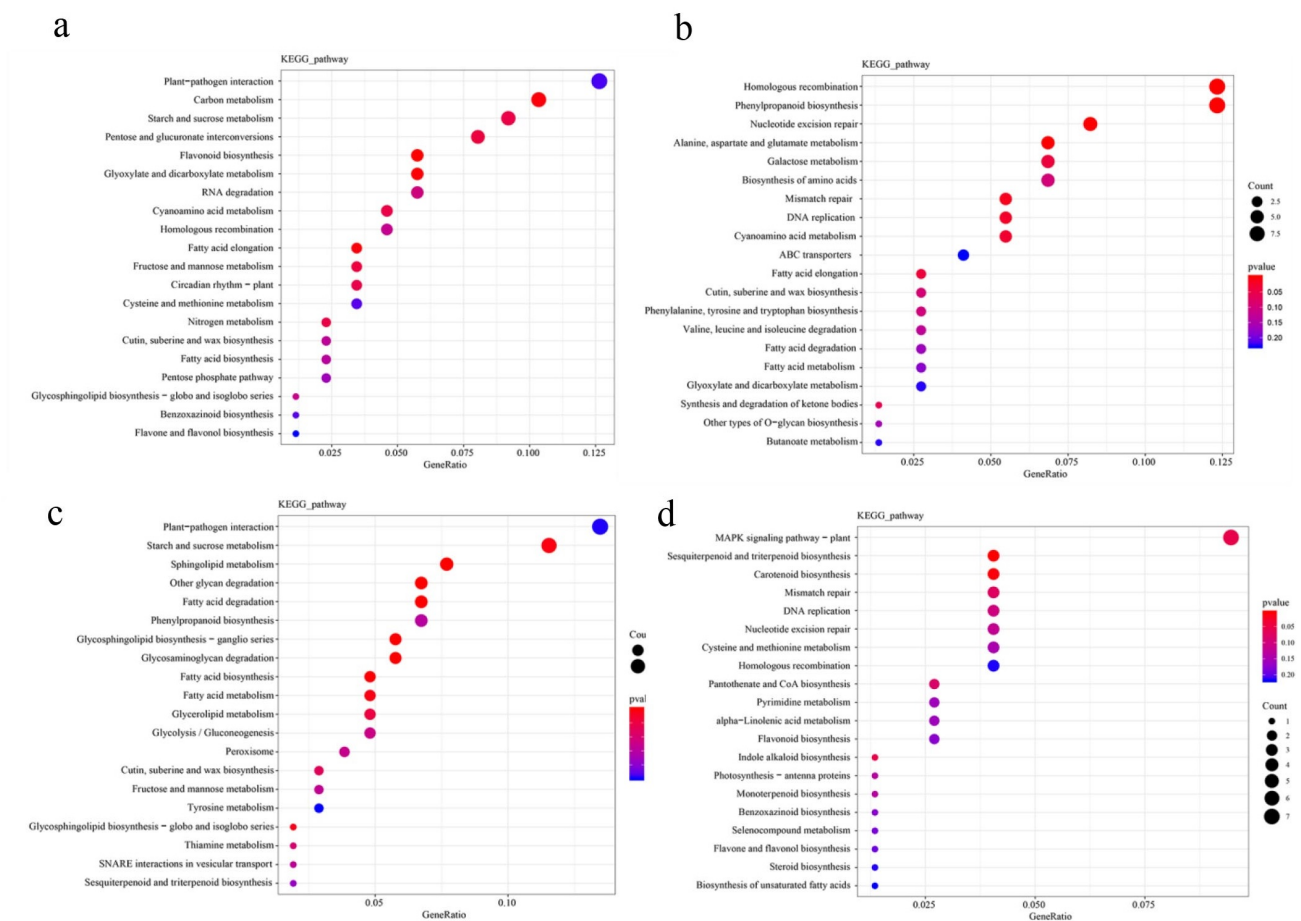
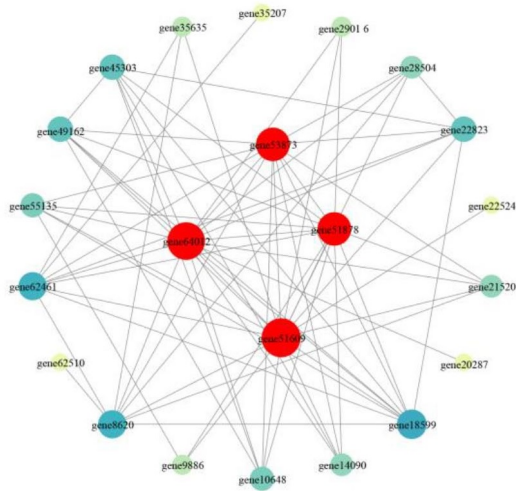


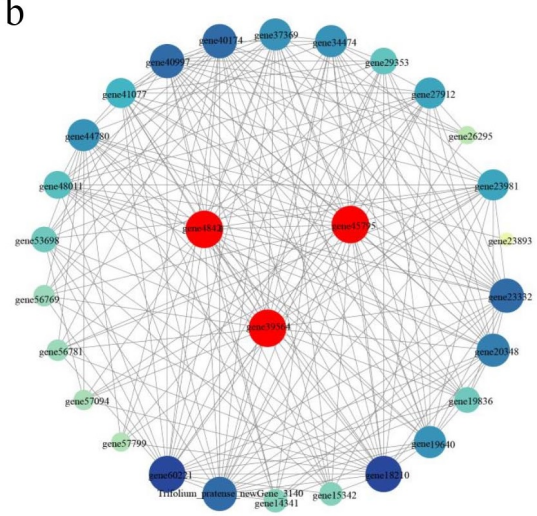
Figure 5

Bubble diagram of KEGG enrichment analysis under (a) cyan, (b) purple, (c) lightcyan and (d) ivory module.

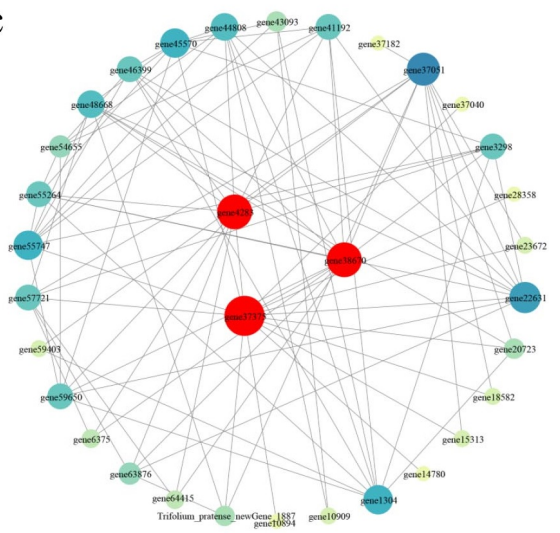
a



b



c



d

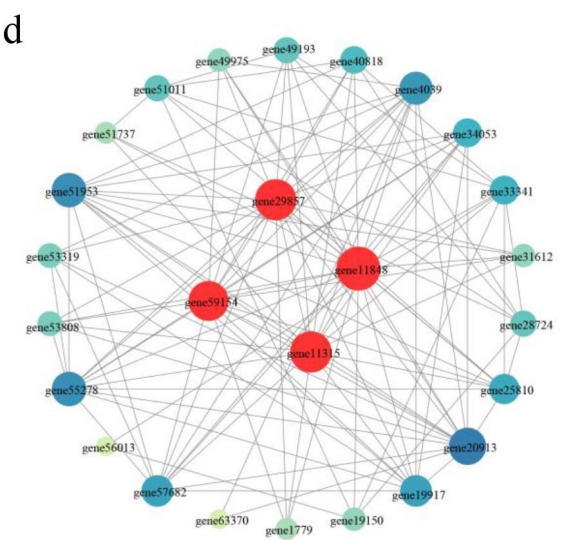


Figure 6

Map of gene interaction network in the target module. (a) Cyan module network interworking diagram. (b) Purple module network interworking diagram. (c) Lightcyan module network interworking diagram. (d) Ivory module network interworking diagram.

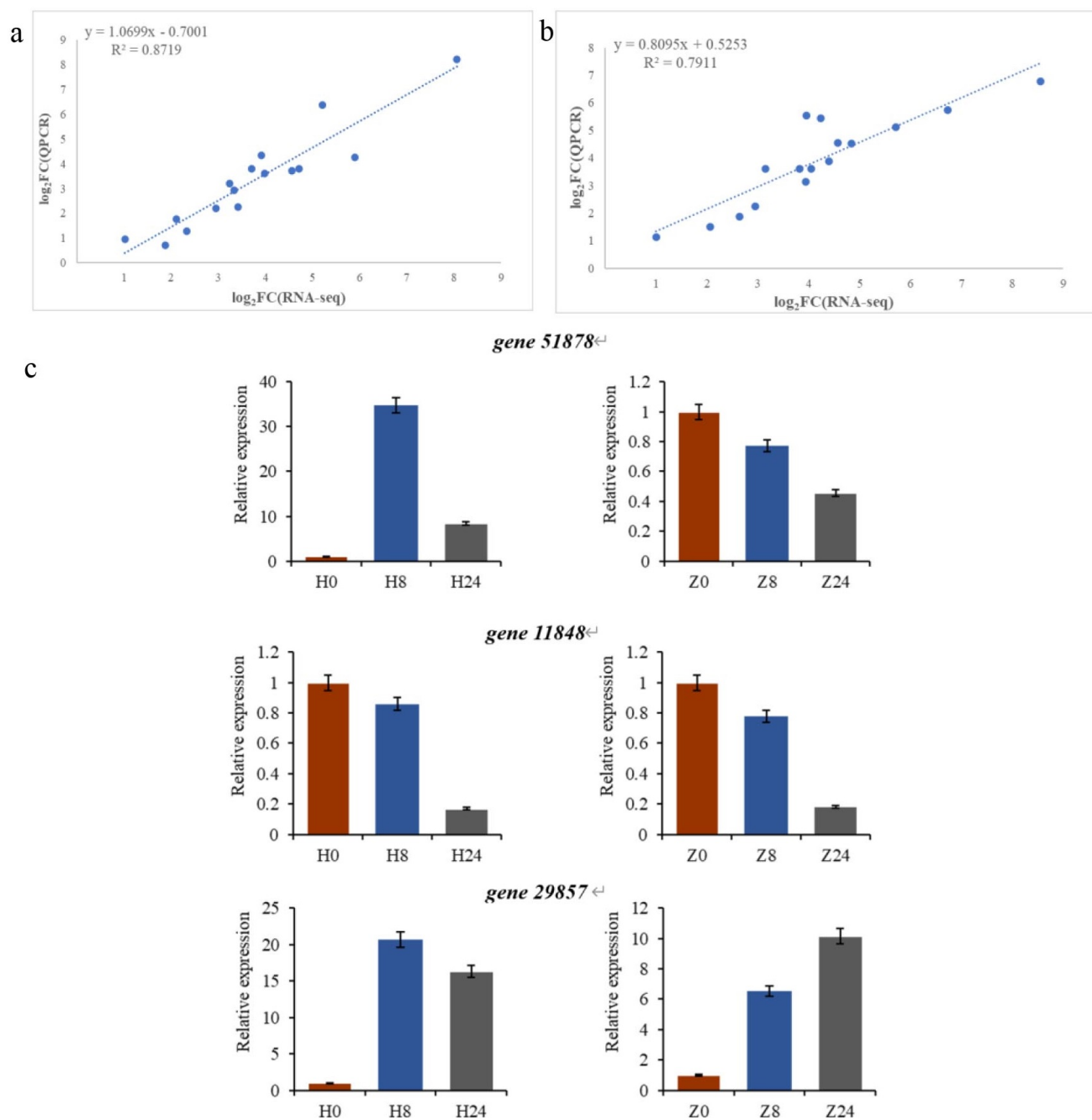


Figure 7

Correlation between RNA-Seq and qRT-PCR gene expression levels in (a) HL and (b) ZY. (c) Hub gene expression level qRT-PCR validation..

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