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# Transcriptome analysis and identification of genes associated with leaf crude protein content in foxtail millet [*Setaria italica* (L.) P. Beauv.]

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**Introduction:** Spruce spider mite is a primary insect pest of Chinese chestnut in China and seriously influences its yield and quality. However, the current management against this mite is costly and poorly effective. In previous research, we bred several foxtail millet materials for interplanting with chestnut tree, and found that they had high levels of crude protein (CP) in leaves and attracted spruce spider mite to feed on the leaves, thereby reducing chestnut damage.

**Methods:** In this study, four foxtail millet varieties with significant differences in leaf crude protein content were used for high-throughput sequencing and identification of genes associated with leaf crude protein content. Gene enrichment analyses were carried out to comprehend the functions of these genes and the biological processes in which they are involved. In addition, transcription factors (TFs) were evaluated.

**Results:** 435 differentially expressed genes (DEGs) were identified, suggesting their potential role in crude protein accumulation. Some differentially expressed genes were found to be associated with nitrogen metabolism and ubiquitin-mediated proteolysis pathways. Moreover, we identified 40 TF genes categorized into 11 transcription factor families.

**Discussion:** Our findings represent an important resource that clarifies the mechanisms of accumulation and control of leaf crude protein in foxtail millet, and provide an opportunity for suppression of spruce spider mite attack on Chinese chestnut by interplanting with foxtail millet varieties with high concentrations of leaf crude protein.

## KEYWORDS

foxtail millet, Chinese chestnut, spruce spider mite, crude protein content, transcriptome analysis

## Introduction

Chestnut has been cultivated in China for more than 2700 years (Adua, 1999). It is an economically valuable plant, its wood used for timber, its leaf and flower utilized as raw material in the pharmaceutical and cosmetic industries, and its fruit peel a source of extracted tannin. Most of all, its fruit is a popular food resource with high nutritional value. The genus *Castanea* comprises 12 world chestnut species, including four cultivated species: Chinese chestnut (*Castanea mollissima* Blume), Japanese chestnut (*Castanea*

*crenata* Sieb. et Zucc.), American chestnut (*Castanea dentata* [Marsh.] Borkh), and European chestnut (*Castanea sativa* Miller) (Jiang et al., 2019). Among these, Chinese chestnut and Japanese chestnut are the most commercially important (Tanaka and Kotobuki, 1992).

Chinese chestnut is commonly grown in China for its fruits. However, several insect pests can seriously damage the tree's trunk, buds, and fruit, and reduce nut production and quality (Gokce et al., 2020). One such pest is the spruce spider mite (*Oligonychus ununguis* Jacobi), a typical polyphagous mite that consumes the chestnut's leaves (Wang et al., 1989; Wang et al., 2010). In the main chestnut-producing region of China, the occurrence of spruce spider mite generally leads to a 15%–50% decline in production (Wang et al., 1991).

To date, several biological and chemical control measures have been taken to prevent this mite from attacking trees in China, including the use of natural enemy species and pesticides (Wang et al., 2010; Zhao and Fan, 2019). Nevertheless, because Chinese chestnut trees are tall and mostly distributed in mountains, it is inconvenient to spray pesticides. Moreover, it often results in the development of resistance to pesticides and environmental pollution, thereby slowing the movement toward green, pollution-free production of crops and restricting the development of the Chinese chestnut industry. In sum, the control measures for spruce spider mite prevention are currently expensive and poorly effective.

In previous work, we developed a number of foxtail millet (*Setaria italica* (L.) P. Beauv.) materials for interplanting with chestnut. They were early-maturing, shade-tolerant, could thrive beneath Chinese chestnut trees, realizing the high-efficient utilization of land resources under the trees. Surprisingly, we discovered that the leaves of these materials were attacked by spruce spider mites. This decreased the rate of bad chestnut nuts resulting from mite attack by 5%–15%, while damage to the millet was minimal (Li et al., 2022). Furthermore, these materials had a high content of crude protein (CP) in their leaves.

Plant–insect interactions are intricate and dynamic. To overcome insect attack, plants develop different defense mechanisms, including physical and chemical defenses (War et al., 2012; Belete, 2018). The first mechanical barrier to feeding is formed by structural features on the leaf surface, such as a waxy cuticle, spines and thorns, trichomes, and thick and lignified cell walls. Secondary metabolites, including phenolics, flavonoids, tannins, and lectins, provide the next barriers of protection. Among chemical constituents, nutrients such as amino acids, vitamins, proteins, and carbohydrates have been also reported to affect the relationship between plants and insects (Li and Jin, 2005; Yang et al., 2014; Li and Li, 2018). In one study, nymphal density and the leaf damage index of grape leafhopper (*Erythroneura apicalis* Nawa) were negatively correlated with CP levels in grape leaf (Fan et al., 2008). Another study reported that total soluble protein content in cotton leaves was positively associated with the population densities of thrip (*Thrips tabaci*), whitefly (*Bemesia tabaci*), and jassid (*Amrasca devastans*), and these pests were more prone to attack cotton genotypes with high concentrations of such proteins (Rizwan et al., 2021). Hence, the protein content of host plant leaves may be related to the degree of insect infestation, which could explain the effect of the foxtail millet materials we bred in previous work. Consequently, it is important to mine genes related to CP accumulation in foxtail millet leaves and to elucidate their regulation mechanisms. This would be helpful for breeding of foxtail millet varieties with high concentrations of leaf CP, and provide an effective, economically, and environmentally safe control measure for spruce spider mite through interplanting.

In this study, we performed transcriptome analyses on four foxtail millet varieties with significantly different leaf CP levels to identify genes associated with leaf CP content. We identified and characterized differentially expressed genes (DEGs) and conducted gene enrichment analyses to investigate their possible functions. We also predicted differentially expressed transcription factor (TF) genes. Our results lay a foundation for clarifying the biological processes and regulation mechanisms underlying leaf CP accumulation, and provide potentially precious gene resources for the future development of foxtail millet varieties with high concentrations of leaf CP.

## Materials and methods

Below we provide a summary of our methods. More details can be found in the Supplementary Materials and Methods.

### Plant materials

Four hybrid varieties of foxtail millet (51950, 12950, 1121, 57295) and a conventional variety (JG32) grown in an experimental field of Beiguan Village, Tangshan, China (40°10'N, 118°28'E) were utilized. The millet was sown in 10 m long rows with 0.5 m between rows. For each variety, three standard plants were chosen, and the middle parts of the first, second, and third leaves from the top of each were collected during the booting stage. Then we mixed and ground the sampled leaves for CP content analysis, RNA-seq analysis, and quantitative real-time PCR (qRT-PCR) validation.

### Chemical analysis of crude protein concentration

Hebei Jintianfeng Grain Trading Co., Ltd. (China) conducted the CP concentration analysis. Total nitrogen content was obtained using the Kjeldahl method (Kjeldahl, 1883), and the value was multiplied by 6.25 to calculate CP content. Each sample had three biological replicates.

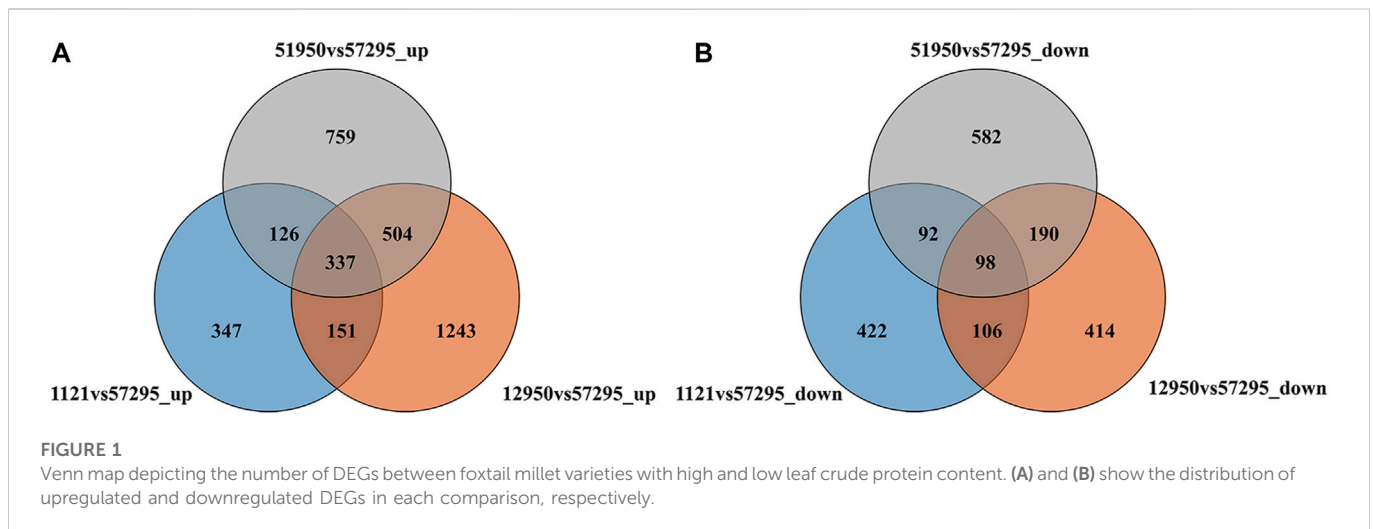
To screen varieties with different CP levels, JG32 was used as a control, which has been found to be suitable for use in interplanting with Chinese chestnut. Three varieties with high CP concentrations (51950, 12950, and 1121) and one with a low concentration (57295) were selected for high-throughput sequencing. The CP levels are listed in Supplementary Table S1.

### Library construction, high-throughput sequencing, and transcriptome analysis

To mine genes associated with leaf CP content, the leaves of each variety were sampled as mentioned above. RNA extraction, library construction, high-throughput sequencing, and transcriptome assembly were carried out as described previously (Li et al., 2022).

### Screening of differentially expressed genes

Fragments per kilobase of transcript per million fragments mapped (FPKM) values were used to determine the gene



expression levels (Trapnell et al., 2010). To detect DEGs, the software edgeR version 3.3.3 (Robinson et al., 2009) was used, and genes with a  $|\log_2(\text{fold change})|$  value  $>1$  and adjusted  $p$ -value ( $\text{padj}$ )  $< 0.05$  were considered significant DEGs.

## qRT-PCR validation

The qRT-PCR validation was conducted as described previously (Liu et al., 2021). Three independent replicates were analyzed, and the primers used are listed in Supplementary Table S2.

## Functional enrichment analyses

GO functional enrichment analysis and KEGG pathway analysis of DEGs were conducted using topGO (Yu et al., 2010) and KOBAS 3.0 (Xie et al., 2011), respectively.

## Identification of TFs

The online webserver PlantTFDB 5.0 (<http://planttfdb.gao-lab.org/prediction.php>) with default parameters was used to identify TF families (Jin et al., 2017). In this tool, ESTScan 3.0 was employed to analyze coding regions in the provided input sequences (Iseli et al., 1999). A heatmap of differentially expressed TF genes was generated using TBtools software (Chen et al., 2020).

## Results

### Transcriptome sequencing and assembly

To characterize genes associated with biosynthesis and accumulation of leaf CP, 12 cDNA libraries from leaf samples of the four varieties with different CP levels were used for RNA sequencing (three library repeats for each variety). Approximately 645.17 M raw reads were generated using an Illumina Novaseq

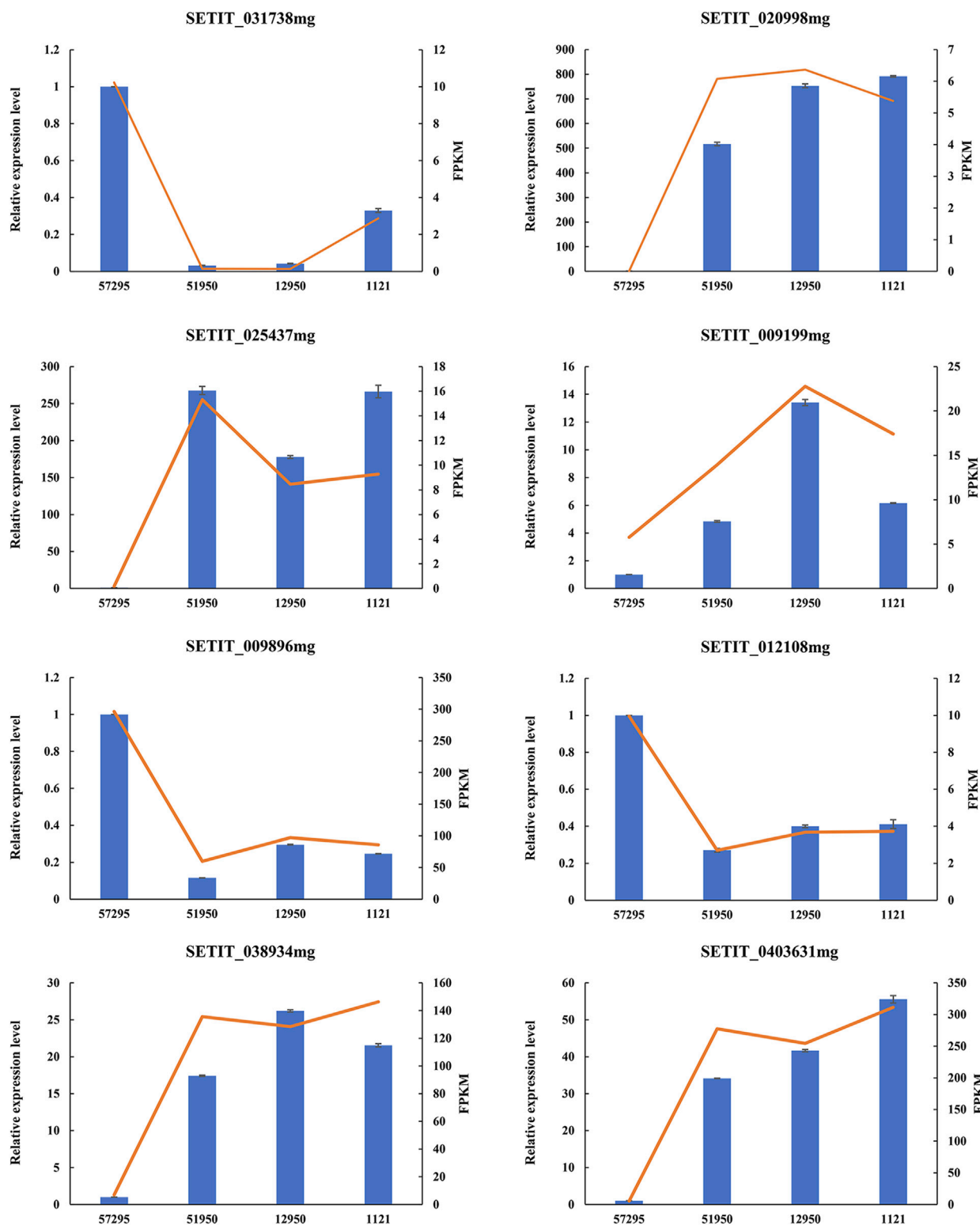
PE150 platform. After quality control, we obtained 641.29 M clean reads and found that 94.76%–95.65% were mapped to the foxtail millet reference genome (*Setaria italica*\_v2.0, [http://plants.ensembl.org/Setaria\\_italica/Info/Index](http://plants.ensembl.org/Setaria_italica/Info/Index)), provided in a previous report (Liu et al., 2022). Furthermore, Pearson's correlation coefficients between biological replicates showed that the biological replicates were highly correlated (Liu et al., 2022), indicating the reliability of the RNA-seq results for gene expression analysis. In total, 30,141 genes were discovered. The FPKM values for each gene were computed and are listed in Supplementary Table S3.

### Identification of differentially expressed genes

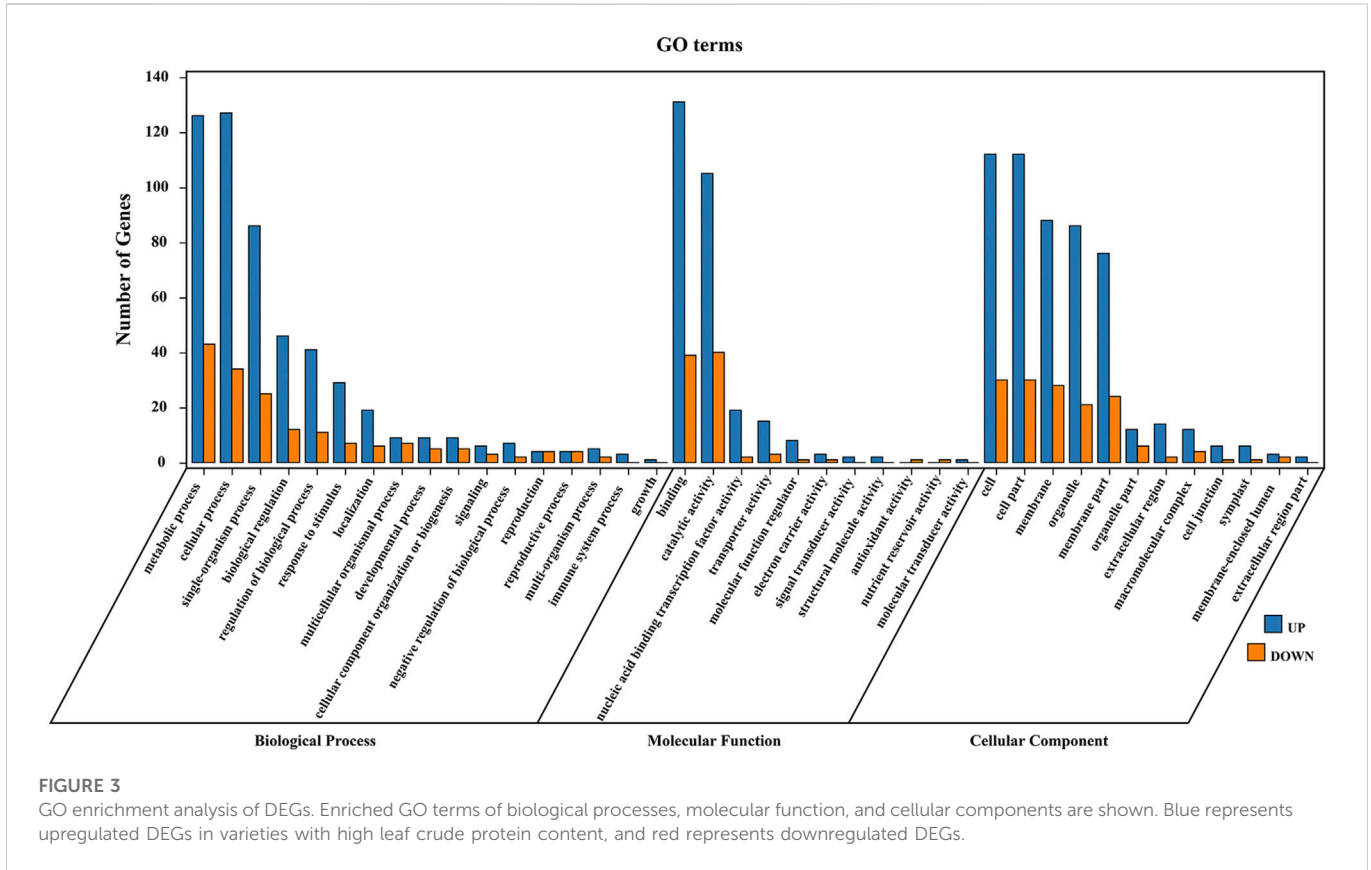
As shown in Figure 1, compared to the low-CP variety 57295, 1726, 2235, and 961 DEGs were upregulated in the high-CP varieties 51950, 12950, and 1121, respectively. In all, 3467 upregulated DEGs were identified, 337 of which were shared among the three high-CP varieties. Likewise, 962, 808, and 718 DEGs were downregulated in 51950, 12950, and 1121, respectively. In all, 1904 downregulated genes were identified, among which 98 were shared. Differential expression analysis provided 435 common DEGs shared in all comparisons, representing possible candidate genes associated with CP accumulation in foxtail millet.

### Validation of differentially expressed genes using qRT-PCR

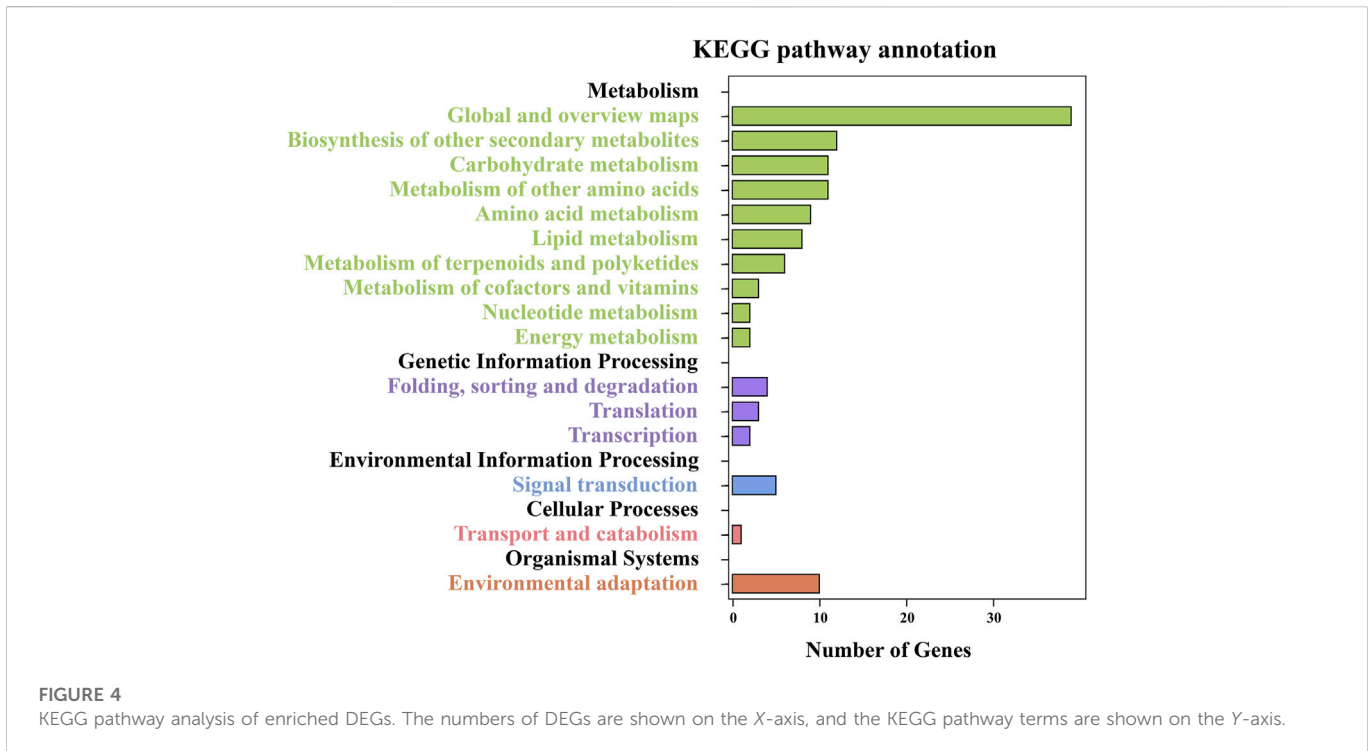
To verify the accuracy of the RNA-seq data, the relative expression levels of eight randomly selected DEGs were examined *via* qRT-PCR. These eight DEGs included five genes that were predicted to be upregulated in varieties with high CP concentrations (SETIT\_020998mg, SETIT\_025437mg, SETIT\_009199mg, SETIT\_038934mg, SETIT\_0403631 mg), and three genes predicted to be downregulated (SETIT\_031738mg, SETIT\_009896mg, SETIT\_012108 mg). As shown in Figure 2, the expression profiles were in line with the RNA-seq data, further demonstrating the reliability of our RNA-seq data.



**FIGURE 2** Validation of eight DEGs by qRT-PCR. The X-axis represents foxtail millet varieties with different levels of leaf crude protein content, and the Y-axis (left side) indicates the relative expression level of selected genes determined by qRT-PCR (blue columns). The *SiACTIN* gene was used as an internal control, and the transcript level of genes in variety 57295 was set as 1.0. Error bars represent standard error ( $n = 3$ ). The Y-axis (right side) depicts the expression level of genes in RNA-seq data (evaluated by FPKM, red lines).



**FIGURE 3** GO enrichment analysis of DEGs. Enriched GO terms of biological processes, molecular function, and cellular components are shown. Blue represents upregulated DEGs in varieties with high leaf crude protein content, and red represents downregulated DEGs.



**FIGURE 4** KEGG pathway analysis of enriched DEGs. The numbers of DEGs are shown on the X-axis, and the KEGG pathway terms are shown on the Y-axis.

## Gene enrichment analysis of differentially expressed genes

For 435 common DEGs in all comparisons, GO category enrichment analysis was conducted using topGO (Yu et al., 2010) to determine their potential biological processes and functions (Supplementary Table S4). These DEGs were annotated to three categories and 40 GO terms: 42.5% of the terms were classified as biological processes, 27.5% were molecular functions, and 30% were cellular components (Figure 3). In the biological process category, genes were most enriched in metabolic processes (GO:0008152) and cellular processes (GO:0009987). In the molecular function category, most genes fell into the binding (GO:0005488) and catalytic activity (GO:0003824) subgroups. In the cellular component category, the genes mainly belonged to cell (GO:0005623) and cell part (GO:0044464) subgroups.

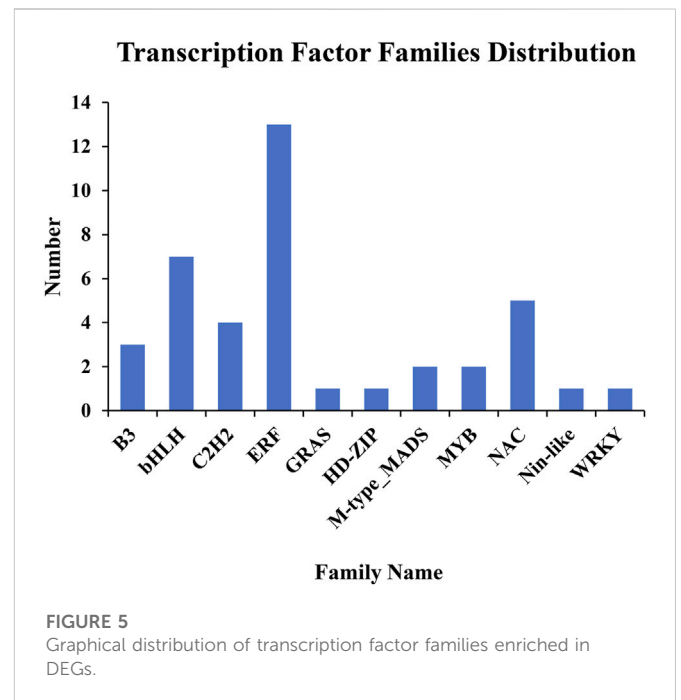
To further understand the functional categorizations, the 435 DEGs were also subjected to KEGG enrichment analysis (Table S5); 174 DEGs were assigned to 60 enrichment pathways categories, of which the top five were metabolic pathways (35 genes, 20.11%), biosynthesis of secondary metabolites (28, 16.09%), plant-pathogen interactions (8, 4.60%), cyanoamino acid metabolism (7, 4.02%), and phenylpropanoid biosynthesis (7, 4.02%). These pathways were classified into five main categories and 16 subcategories, with the top five subcategories being global and overview maps, biosynthesis of other secondary metabolites, carbohydrate metabolism, metabolism of other amino acids, and environmental adaptation (Figure 4). The enrichment analysis illustrated that changes in leaf CP content significantly impacted the life processes of foxtail millet.

Amino acids are necessary for the formation of proteins and other related compounds, with nitrogen being the essential constituent (Mokhele et al., 2012). Due to the close relationship between nitrogen and protein and other nitrogenous compounds, nitrogen metabolism in plants directly affects CP content. According to the KEGG pathway analysis, the genes SETIT\_014128 mg and SETIT\_014124 mg present in carbonic anhydrase (EC:4.2.1.1) were enriched in the nitrogen metabolism pathway and both were upregulated in leaf tissues of foxtail millet varieties with high CP concentrations.

As one of the most prevalent protein posttranslational modifiers, ubiquitin (Ub) is a small peptide of 76 amino acids that can be attached to target proteins *via* ubiquitination (Hershko et al., 1998). Protein ubiquitination is involved in the regulation of proteolysis, subcellular localization, and the stability and activity of substrate proteins (Zhou et al., 2017). As per the KEGG analysis, two genes, SETIT\_004756 mg present in E3 ubiquitin-protein ligase SIAH1 (EC:2.3.2.27) and SETIT\_025290 mg present in ubiquitin-conjugating enzyme E2 J2 (EC:2.3.2.23), were annotated to ubiquitin-mediated proteolysis, a major pathway of degradation of cellular proteins. Based on differential expression analysis, SETIT\_004756 mg and SETIT\_025290 mg were downregulated and upregulated in varieties with high CP concentrations, respectively.

## TF analysis

The GO enrichment analysis revealed that several DEGs may function in regulation of transcription and have TF activity (Supplementary Table S4). TFs are DNA-binding proteins that

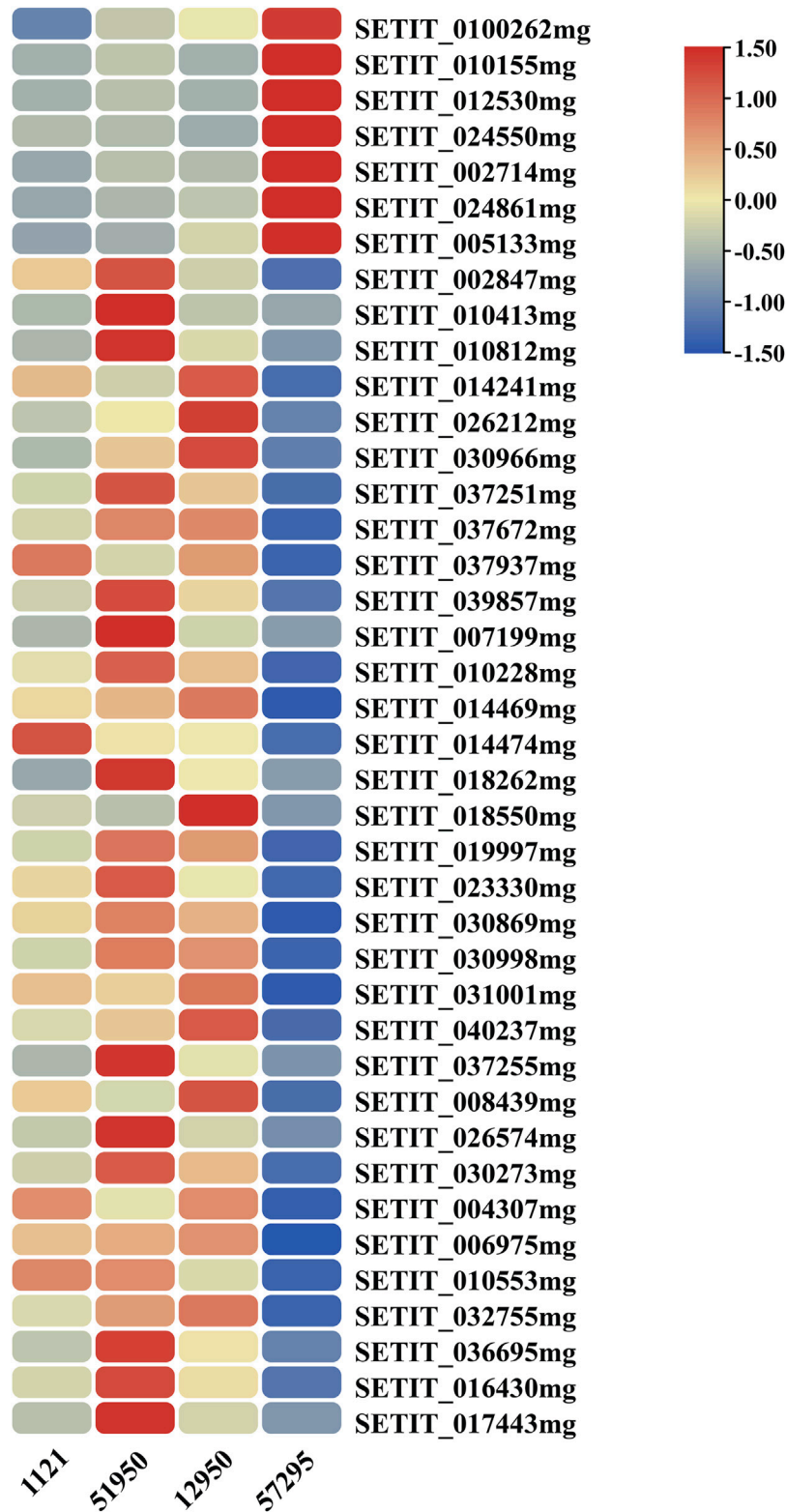


mediate many processes by playing a crucial role in gene transcription and expression. As a consequence, to more fully understand the functions of DEGs and analyze their regulatory mechanisms, we predicted possible TF genes from among the 435 common DEGs shared in all comparisons using the plant TF database PlantTFDB (Jin et al., 2017). In total, 40 genes were identified, including 33 upregulated genes and seven downregulated genes, and they were grouped into 11 different TF families (Figure 5; Figure 6, Table S6). Among them, the ERF family was the most abundant family (13 genes). The remaining top 4 TF families were bHLH (seven genes), NAC (five genes), C2H2 (four genes), and B3 (three genes).

## Discussion

In the natural environment, plants are constantly threatened by herbivorous insects. For Chinese chestnut, damage from spruce spider mite is a primary challenge restricting its output and quality, and generally leads to a serious commercial loss. In previous work, we developed several shade-tolerant foxtail millet varieties for interplanting with chestnut trees, and found that it improved the quality of chestnut fruits by attracting spruce spider mite to feed on their leaves. This strategy could become a new, effective, and environmentally friendly, pest-management option for chestnut tree.

Nutrients of host plants, such as proteins and carbohydrates, are essential sources of energy for the growth and development of herbivorous insects. CP content in leaves affects the lifespan, development duration, and egg laying outcomes of mites, and in certain plant species the degree of leaf damage by insects is higher for leaves with higher CP levels (Wu et al., 1995; Fan et al., 2008; Wei et al., 2009; Yang et al., 2014; Sonalkar, 2020). The foxtail millet varieties we previously bred for interplanting with chestnut tree all had high CP levels in leaves, which may be one major reason why they attracted the spider mites. In present study, four foxtail millet varieties with significant differences in leaf CP



**FIGURE 6**  
Heatmap of the differentially expressed transcription factor genes. The color gradient indicates the FPKM value of genes; red and blue represent high and low expression, respectively.

content were used to identify genes related to CP levels via transcriptome sequencing technology. We successfully identified 435 DEGs, including 337 upregulated DEGs and 98 downregulated DEGs in varieties with high CP

concentrations. Our findings provide a precious resource for research on the mechanisms that control the accumulation of CP, and offer a chance to effectively control spruce spider mite outbreaks among chestnut trees.

Protein is an important product of nitrogen metabolism, and therefore CP levels are closely associated with nitrogen metabolism. For example, an increase in CP levels of vegetable leaves is correlated with an increase in N uptake (Mhlontlo et al., 2007). Most plants assimilate inorganic and organic forms of nitrogen, including nitrate and ammonium (Temple et al., 1998). Metabolism normally begins with nitrate being converted into nitrite in the presence of nitrate reductase, and then the nitrite is reduced to form ammonia by nitrite reductase. Following its formation, ammonia is assimilated by a number of pathways, including reductive amination and transpiration, to yield a variety of amino acids (Mokhele et al., 2012; Kishorekumar et al., 2020). In the current study, two DEGs, SETIT\_014128 mg and SETIT\_014124mg, were enriched in the nitrogen metabolism pathway and may code for carbonic anhydrase (CA). The reversible conversion of carbon dioxide to bicarbonate ( $\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{HCO}_3^- + \text{H}^+$ ) is catalyzed by this enzyme in the leaves of  $\text{C}_4$  plants (Ludwig, 2016). Cyanate in plants, which is derived from endogens cyanide or comes from the environment, is decomposed into carbon dioxide and ammonia ( $\text{CNO}^- + \text{HCO}_3^- + 2\text{H}^+ \rightleftharpoons \text{NH}_3 + 2\text{CO}_2$ ), and produced ammonia enters into biosynthesis pathway of amino acids. This reaction is bicarbonate-dependent (Johnson and Anderson, 1987; Kozliak et al., 1995; Ebbs, 2004; Yu et al., 2019). Thus, SETIT\_014128 mg and SETIT\_014124 mg may promote nitrogen metabolism and subsequent biosynthesis of proteins by upregulating their expression level and the activity of CA. In addition, in the first step of  $\text{C}_4$  photosynthesis, phosphoenolpyruvate carboxylase (PEPC) uses bicarbonate as a substrate, whose generation from carbon dioxide is catalyzed by CA (Ludwig, 2016); the decrease in CA accumulation and CA activity plays a considerable role in protein accumulation of PEPC (Chatterjee et al., 2021). In addition to having a significant impact on carbon and nitrogen metabolism, PEPC also influences protein synthesis by promoting the distribution of the carbon skeleton to amino acid production (Shinano et al., 2006). One study found that applying the nanobiomaterial ‘nanochitin whisker’ primarily facilitated the accumulation of nitrogen and translocation more than carbon metabolism and enhanced CP concentration in grain by stimulating PEPC activity and reducing the ratios of sucrose phosphate synthase (SPS) to PEPC in flag leaves and spikes of wheat during the anthesis and grain-filling stages (Cheng et al., 2019), revealing the role of PEPC in the coordination of carbon and nitrogen flow to amino acids and carbohydrates. These findings further support that SETIT\_014128 mg and SETIT\_014124 mg may be hub genes associated with leaf CP concentration in foxtail millet, and can be used to develop high-CP varieties by genetic manipulation; however, their detailed functions need to be further clarified.

In eukaryotes, the ubiquitin-proteasome system (UPS) is responsible for the majority of cytosolic protein breakdown, accomplished through the degradation of the substrate protein by ATP-dependent 26S proteasome after covalent attachment of many Ub molecules to the substrate protein (Ciechanover et al., 2000; Nandi et al., 2006). Binding of Ub to target proteins includes the following steps. First, the C-terminal Gly of Ub is activated and transferred to a carrier E2 protein (Ub-conjugating enzyme) by the Ub-activating enzyme E1. Then with the aid of E3 (a Ub-protein ligase enzyme), the E2 protein tags Ub to the target protein. Various E3 ligases within the UPS recognize substrates carrying

diverse degradation signals, giving the system high specificity and selectivity. In this study, we identified two DEGs that encode Ub-conjugating enzyme E2 J2 (UBE2J2) and E3 Ub-protein ligase SIAH1, namely, SETIT\_025290 mg and SETIT\_004756mg, respectively. The UBE2J2 protein is a Ub-conjugating enzyme which, in association with Ub ligases including TEB4, PARKIN, CHIP, and cIAP1, promotes proteasomal degradation in mammals and responds to proteotoxic stress (Imai et al., 2002; Lenk et al., 2002; Kim et al., 2003; Wu et al., 2005; Choi et al., 2010; Claessen et al., 2010; Burr et al., 2011). Nevertheless, no UBE2J2 proteins have been characterized in plants to date. The seven in absentia (SINA)/seven in absentia homolog (SIAH) family proteins are E3 Ub-protein ligases with a RING domain and are highly conserved from plants to mammals (Polekhina et al., 2002). This superfamily was first characterized in *Drosophila melanogaster* and plays a crucial role in the regulation of development and proteasome-mediated protein degradation (Hu and Fearon Eric, 1999). To date, 18 SINA proteins homologous to the *Drosophila* SINA protein have been defined in *Arabidopsis*, among which SINAT5 targets the noapical meristem/cup-shaped cotyledon 1 (NAC1) TF and late elongated hypocotyl (LHY) for degradation and plays a role in various developmental processes in plants (Xie et al., 2002; Park et al., 2010). Therefore, SETIT\_025290 mg and SETIT\_004756 mg may be involved in specific ubiquitination and subsequent proteolysis of proteins, and have an impact on leaf CP content in foxtail millet by regulating protein levels and the stability of related proteins. However, their biological functions and regulation mechanisms remain to be elucidated in future work.

TFs regulate a variety of biological processes, such as plant metabolism, growth, and development, by binding to the promoters (or intragenic regions) of target genes (Kaufmann et al., 2018; Burgess et al., 2019). Many TFs have been found to control nitrogen assimilation. Recently, Wei et al. reported that overexpression of the *dehydration-responsive element-binding protein 1C* (*OsDREB1C*) gene in rice (*Oryza sativa*), a member of the AP2/ERF TF family, improved nitrogen content, nitrogen use efficiency (NUE), and protein abundance in leaves by elevating nitrogen uptake and transport activity (Wei et al., 2022). Furthermore, a few TFs affect protein synthesis in plants. For example, overexpressing the MYB family TF *TaODORANT1* in common wheat (*Triticum aestivum*) and its homolog in *Triticum urartu* reduces the transcription levels of seed storage protein (SSP) genes and total SSP levels of mature grains, revealing an inhibition effect on SSP synthesis (Luo et al., 2021). *OPAQUE 11* (*O11*) encodes a seed-specific bHLH TF in maize, and the protein levels of loss-of-function mutant *o11* endosperm per kernel are significantly decreased compared to wild type (Feng et al., 2018), revealing regulatory role in protein accumulation. In addition, several NAC TFs have also been reported to regulate protein accumulation and content in grains of wheat, rice, and maize (*Zea mays*) (Uauy et al., 2006; Liang et al., 2014; Zhang et al., 2019; Wang et al., 2020). An endosperm-specific NAC TF, *TaNAC019*, modulates SSP accumulation in wheat seeds (Gao et al., 2021). It directly binds to the promoters of *high-molecular-weight glutenin* (*HMW-GS*) genes and activates their expression, and triple knock-out mutants of *TaNAC019* homologs have lower gluten levels. In the current research, 40 DEGs were identified as TF genes, containing several members of the bHLH, MYB, NAC, and especially ERF gene families (Figure 5). It can be speculated that these genes may function as TFs and improve CP accumulation in leaves of



foxtail millet by modulating nitrogen assimilation and protein synthesis. However, the detailed underlying molecular mechanisms involved require further research, and will be a focus of ours in the future.

## Conclusion

Transcriptome analysis of genes in foxtail millet associated with CP accumulation in leaves was performed and 435 DEGs were identified. Several DEGs related to nitrogen metabolism and ubiquitin-mediated proteolysis pathways were characterized. We also predicted the TFs that participate in the control of CP levels. These findings provide a resource that clarifies the accumulation and control mechanisms of CP levels in leaves. This information can be used to develop foxtail millet varieties with high CP concentrations for interplanting with chestnut, to improve production of this important crop.

## Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA772942>.

## Author contributions

The research was conceived and designed by ZL and SL. DL, ZZ, SL, YC, and JZ performed the experiments. Data was analyzed by DL, ZZ, and YC. YC wrote the original draft. ZL and DL made revisions to

the final manuscript. The final manuscript was read and approved by all authors.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fgene.2023.1122212/full#supplementary-material>

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