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Transcriptome Analysis of Hybrid Progeny of Caucasian Clover and White Clover in the Early Stages of Rhizobia Infection

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Abstract: The hybrid progeny (1-1) resulting from the cross between Caucasian clover and white clover initially demonstrated an inability to fix nitrogen naturally via spontaneous nodulation. However, following inoculation with specific rhizobia strains derived from the Trifolium genus, successful nodulation and nitrogen fixation were observed in the 1-1 progeny, resulting in enhanced biomass production and adaptability. To explore in greater depth the mechanisms driving nitrogen fixation in these hybrid progeny, the inoculation was carried out using the dominant rhizobia strain (No. 5), isolated from Mengnong Clover No. 1. Root samples were collected at 3, 6, and 9 days post inoculation for RNA sequencing. A total of 1755 differentially expressed unigenes were identified between the control and treatment groups. KEGG pathway analysis highlighted key pathways associated with nodule nitrogen fixation. In combination with Weighted Gene Co-expression Network Analysis (WGCNA) and Gene Set Enrichment Analysis (GSEA), several differentially expressed genes were identified, suggesting their potential contribution to nitrogen fixation. Noteworthy among these, the gene *TRINITY_DN7551_c0_g1* in the Phenylpropanoid biosynthesis pathway (MAP00940) emerged as a key candidate. This study offers valuable RNA-seq data, contributing significantly to the understanding of the molecular regulatory mechanisms underpinning nodule nitrogen fixation in legumes, thereby laying a solid foundation for future investigations into the hybrid progeny of Caucasian and white clover crosses.

Keywords: Caucasian clover; transcriptome; differentially expressed genes; nodular nitrogen; rhizobia

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Academic Editor: Jun Zhou

Received: 2 January 2025
Revised: 6 February 2025
Accepted: 19 February 2025
Published: 27 February 2025

Citation: Zhu, P.; Wang, S.; Cao, K. Transcriptome Analysis of Hybrid Progeny of Caucasian Clover and White Clover in the Early Stages of Rhizobia Infection. *Nitrogen* **2025**, *6*, 11. https://doi.org/10.3390/nitrogen6010011

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1. Introduction

The legume family is distinguished among plants by its unique ability to form symbiotic relationships with soil-dwelling bacteria known as rhizobia [1]. This symbiosis results in the establishment of nitrogen-fixing root nodules, specialized structures that develop on the plant root. Within these nodules, rhizobia are colonized and facilitate the reduction of atmospheric nitrogen into ammonia, a process referred to as Biological Nitrogen Fixation (BNF) [2]. The ammonia produced is subsequently assimilated by the host plant to support its growth, while the rhizobia receive photosynthetically derived carbon in the form of dicarboxylic acids as a reciprocal carbon source [3]. Because of the high specificity of symbiosis between legumes and rhizobia [4], host specificity is largely determined by the flavonoid produced by the plant and NFs produced by rhizobia [5,6].

Caucasian clover (*Trifolium ambiguum*) is an excellent perennial legume forage grass, native to the Caucasus region, capable of adapting to different soil types and with good

tolerance to drought, cold, frequent mowing, and grazing [7], with an extremely well-developed primary root and a huge underground root-tiller system [8]. The most important economic value of leguminous forage grasses lies in their ability to intercrop with rhizobia for symbiotic nitrogen fixation. However, Caucasian clover cannot nodulate and fix nitrogen under natural conditions in non-native areas [9], and most of the rhizobia formed by it is ineffective [10]. In response to this deficiency, some researchers have attempted to improve its traits by crossing it with white clover and making the hybrid progeny inherit the nitrogen fixation capacity of white clover for nitrogen fixation [11]. It is worth mentioning that in 2012, Inner Mongolia Agricultural University successfully bred China's first new Caucasian clover variety "Mengnong Clover No. 1" (*T. ambiguum* Bieb. cv. Mengnong No. 1), which can nodulate and fix nitrogen under natural conditions. The forage breeding team of Inner Mongolia Agricultural University also used Caucasian clover and white clover to cross and obtain a large number of hybrid progeny through embryo rescue culture technology, and after years of selection and breeding, a series of hybrid progeny lines were formed. However, they did not inherit the characteristics of white clover's strong nitrogen fixation ability and still could not nodule nitrogen fixation under natural conditions.

Transcriptome sequencing (high-throughput RNA sequencing, RNA-seq) allows the detection of all genes that are differentially expressed during symbiosis, such as the more pronounced transcriptional level response that occurred after soybean was inoculated with two different genera of rhizobia (slow-growing rhizobia *japonicum* and Chinese rhizobia *fischeri*). Considering the complexity of the symbiotic process, the dynamic transcriptome analysis of the nodulation process is particularly important. Niyikiza et al. [12] analyzed rhizobia-infested root nodules at 12, 22, and 36 d. The study identified 9669 genes that are continuously regulated and identified key genes that influence nodulation by regulating the synthesis of secondary metabolites. For soybean roots, Hayashi et al. [13] used transcriptomics to analyze the infested region of soybean roots infested with wild rhizobia and *nodc*-mutant strains for 2 d. A total of 2915 genes regulated by rhizobia infestation were identified, which contained a series of genes related to gibberellin biosynthesis. Carvalho et al. [14] analyzed soybean root material infested with rhizobia for 10 d by transcriptome analysis and identified 3210 genes significantly regulated by rhizobia infestation, and by enrichment analysis, the regulated genes were found to be mainly involved in metabolic processes as well as stimulus response regulation.

Transcriptomic studies on the progeny of crosses between Caucasian clover and white clover inoculated with rhizobia have not been reported. Here, we report the comparative RNA-seq analysis of Caucasian clover and white clover hybrid progeny line 1-1 (hereafter referred to as 1-1). The test strains were five rhizobia isolated from Mengnong clover No. 1, and this study aimed to analyze the gene expression of hybrid progeny line 1-1 at different time points after inoculation with rhizobia in combination with RNA-seq comparison.

2. Materials and Methods

2.1. Collection and Purification of Rhizobia

The sampling area was the planting area of "Mengnong Clover No. 1" in the experimental field of Qinghai University. The sampling area was divided into 3 plots equally, 6 samples were taken from each area by the three-point sampling method, and 18 samples were collected in total. When sampling, the topsoil was removed, 20–30 cm was dug down, and the roots of clover in the flowering stage and the inter-root soil adhering to them were put together in a sterile bag and brought back to the laboratory to pick the nodules. The collected nodules were rinsed well, and those that were large, full, and pink in color were selected and put into sterile bottles. The nodules were removed from the sterile vials, soaked in 0.1% mercury for 5 min, then soaked in 95% alcohol for 1 min, and

finally washed with distilled water three times to ensure that no agent remained. We placed 1~2 sterilized nodules in sterile Petri dishes, crushed them with sterile forceps, squeezed out the juice, added 2 mL sterile water to dilute, and mixed thoroughly to make a bacterial suspension. We took 0.2 mL of bacterial suspension on YMA medium (10 g of mannitol, 0.5 g of dipotassium hydrogen phosphate, 0.2 g of sodium sulfate, 0.05 g of sodium chloride, 0.4 g of yeast paste, 4 mL of RH trace element liquid, 15 g of agar, 1000 mL of distilled water) and spread it evenly with a sterilized glass rod, and put it into a constant temperature incubator at 28 °C. After 48 h of incubation, colonies were grown on the medium. The single colonies with good growth were selected and repeatedly scribed and cultured on YMA medium until the purified strains were obtained, and after isolating the purer single colonies, they were transferred to YMA slant for culturing, and finally, five rhizobia species were screened and stored at 4 °C for backup [15].

2.2. Plant Materials and Growth Conditions

Using 1-1 seed as material, seeds were rubbed through the seed coat with sandpaper and incubated in an incubator (incubator sterilized with 75% ethanol) at 28 °C, 70% relative humidity, 16 h of light and 8 h of dark light for 5 days, then inoculated with rhizobia 1, 2, 3, 5, and 9 (all kept in our laboratory) and transferred to a nutrient bowl containing vermiculite (sterilized) and incubated in an artificial climate chamber (28 °C, 16/8 h day/night cycle, 70% relative humidity). After inoculation, the appropriate amount of nitrogen-deficient modified Hoagland nutrient solution (NS1010-N-50L) was poured daily. Plants were grown under the same growth conditions and RNA was extracted from the roots at 3 d, 6 d, and 9 d after inoculation.

2.3. Plant Growth Phenotype Determination

Clover seedlings from each treatment group were used for plant phenotypic analysis. Similar to the procedure for collecting RNA-seq samples, clover roots were washed and harvested 60 days after inoculation of the nodules. After the determination of plant height, single root weight, and fresh weight of above- and below-ground parts, the plant samples were placed in an envelope in a blast drying oven, dried to constant weight at 60 °C and then used for the measurement of dry weight of above- and below-ground parts.

2.4. RNA Extraction and RNA-Seq Library Construction

Total RNA was extracted from three biological replicates of the root tissue from each treatment using the total RNA extraction kit (Model: R1200) provided by Beijing Solabao & Technology Co., Ltd. (Beijing, China), in accordance with the manufacturer's protocol. RNA integrity was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Poly(A) mRNA was subsequently enriched from 50 μL of total RNA using NEBNext Oligo(dT)25 beads (NEB, Ipswich, MA, USA). The enriched mRNA was then used to construct a cDNA library utilizing the NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, USA), following the manufacturer's guidelines.

2.5. Quantification of Gene Expression Levels and Differentially Expressed Gene (DEG) Analysis

Gene expression levels were quantified as fragments per kilobase of transcript per million fragments mapped (FPKM), calculated using the following formula: FPKM = (cDNA Fragments)/(Mapped Fragments (Millions) × Transcript Length (kd)).

A differential expression analysis between the two groups was conducted using the DESeq2 package [16], which employs a statistical method based on the negative binomial distribution. The resulting *p*-values were adjusted for multiple testing using the Benjamini–Hochberg procedure (Ghosh and Department of Biostatistics and Informatics, Colorado School of Public Health, Aurora, CO 80045, USA, 2020) to control the False Discovery Rate

(FDR). Genes with an adjusted *p*-value of less than 0.01 and a twofold or greater change in expression identified by DESeq2 [16] were considered differentially expressed. Gene expression patterns were visualized using the Multiple Experiment Viewer (MeV) [17].

2.6. DEG Functional Annotation and Enrichment Analyses

Differentially Expressed Genes (DEGs) were assigned functions and annotated using several comprehensive databases: Nr (NCBI non-redundant protein sequences, ftp://ftp.ncbi.nih.gov/blast/db/), Nt (NCBI non-redundant nucleotide sequences, ftp://ftp.ncbi.nih.gov/blast/db/), Pfam (database of homologous protein families, http://pfam.xfam.org/ (accessed on 6 February 2025)), COG (Clusters of Orthologous Groups of proteins, https://www.ncbi.nlm.nih.gov/COG/ (accessed on 6 February 2025)), Swiss-Prot (a manually annotated and reviewed protein sequence database, https://www.uniprot.org/ (accessed on 6 February 2025)), KO (KEGG Ortholog database, http://www.genome.jp/kegg/ (accessed on 6 February 2025)), and GO (Gene Ontology database, http://www.geneontology.org/ (accessed on 6 February 2025)).

2.7. RNA Extraction and RNA-Seq Library Construction

One-Step Real-Time quantitative PCR (RT-qPCR) was carried out to confirm the RNA-seq data from the extracted M. Sativa leaves' total RNA. The experiments were performed using One-Step TB Green PrimeScript RT-PCR Kit II (Takara, Tokyo, Japan) on an ABI 7500 system (Applied Biosystems, Waltham, MA, USA). Primers for One-Step RT-PCR were designed using Primer Premier 5.0 software (Premier, Toronto, ON, Canada). The Caucasian Clover RCD1, PPIL, and NLI2 genes were selected as the endogenous reference [18]. All the primers were synthesized by ruibiotech (Beijing, China) and are listed in Supplementary Table S1. A 20 µL reaction mix was set up containing 50 ng total RNA and 0.4 µM of each primer. The One-Step RT-qPCR analysis was performed on the extracted total RNA from three biological replicates of the leaves of each M. Sativa variety, and each RNA sample was carried out in technical triplicate. The program was set at 42 °C for 5 min, then 95 °C for 10 s, followed by 40 cycles of 95 °C for 5 s, then 60 °C for 34 s. The final melting curve analysis was set at 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. The relative expression changes of the endogenous reference and tested genes were analyzed by the $2^{-\Delta\Delta Ct}$ method [19].

2.8. Statistical Analysis

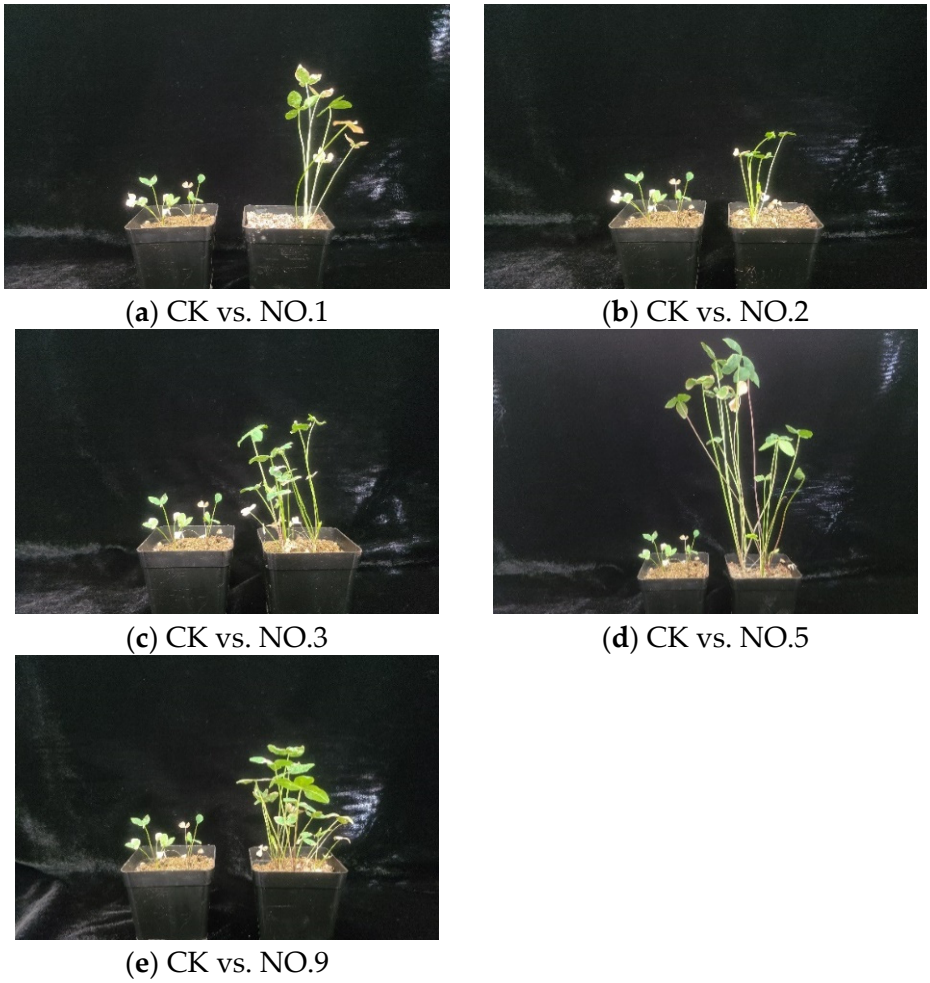
Each treatment was conducted in triplicate, and the results are presented as mean ± standard deviation (SD). The data were analyzed using a one-way analysis of variance (ANOVA). The significance of differences between mean values was determined using the least significant difference (LSD) test at a 0.05 probability level. Microsoft Excel 2010 was used for data entry; IBM SPSS Statistics 20.0 (International Business Machines Corporation, New York, NY, USA) software was used for data collation, principal component analysis, and analysis of variance; and Origin 2021 OriginLab (Northampton, MA, USA) was used for image plotting.

3. Results

3.1. Plant Growth Phenotype Determination

The plants were inoculated with each of the five rhizobia strains, and the uninoculated treatment was used as the control (CK). After 60 days of inoculation, the phenotypes of clover plants were observed for each treatment (Figure 1), indicating that each rhizobial strain treatment significantly promoted the growth of clover plants, with the best effect being the treatment inoculated with bacterium No. 5. Next, we measured the aboveground

biomass dry weight, below-ground biomass dry weight, above-ground biomass fresh weight, below-ground biomass fresh weight, root nodule number, effective root nodule number, and single plant root nodule weight of the plants (Supplementary Figure S1C–H). Compared to the control, the five rhizobial treatments increased above-ground biomass fresh weight, dry weight, root nodule number, effective root nodule number, and single root nodule weight of clover seedlings and all strains except strain 2 significantly increased the below-ground biomass fresh weight and dry weight of clover plants.



(**a**) CK vs. NO.1 (**b**) CK vs. NO.2

(**c**) CK vs. NO.3 (**d**) CK vs. NO.5

(**e**) CK vs. NO.9

Figure 1. Phenotypic variations in the hybrid progeny of Caucasian clover and white clover were assessed under different rhizobial inoculation conditions. Five distinct rhizobial strains were used for inoculation, and several growth parameters—such as plant height, root biomass, and nodule formation—were measured 60 days after the inoculation. Among the strains, strain No. 5 exhibited the most pronounced effect on plant growth, significantly influencing these parameters.

3.2. De Novo Assembly Transcriptome

After inoculation of rhizobia, a total of 114.72 Gb of Clean Data was obtained after transcriptome sequencing of 18 samples, and the sequencing data were assembled using Trinity software, and the assembly yielded a total of 97,066 unigenes, of which there were 29,737 unigenes with a length above 1 kb. The unigenes had an N50 of 1707 and high assembly integrity. The largest number of unigenes was found in the 200–500 interval, with 39,537 or 41% (Supplementary Figure S2).

3.3. Gene Annotation

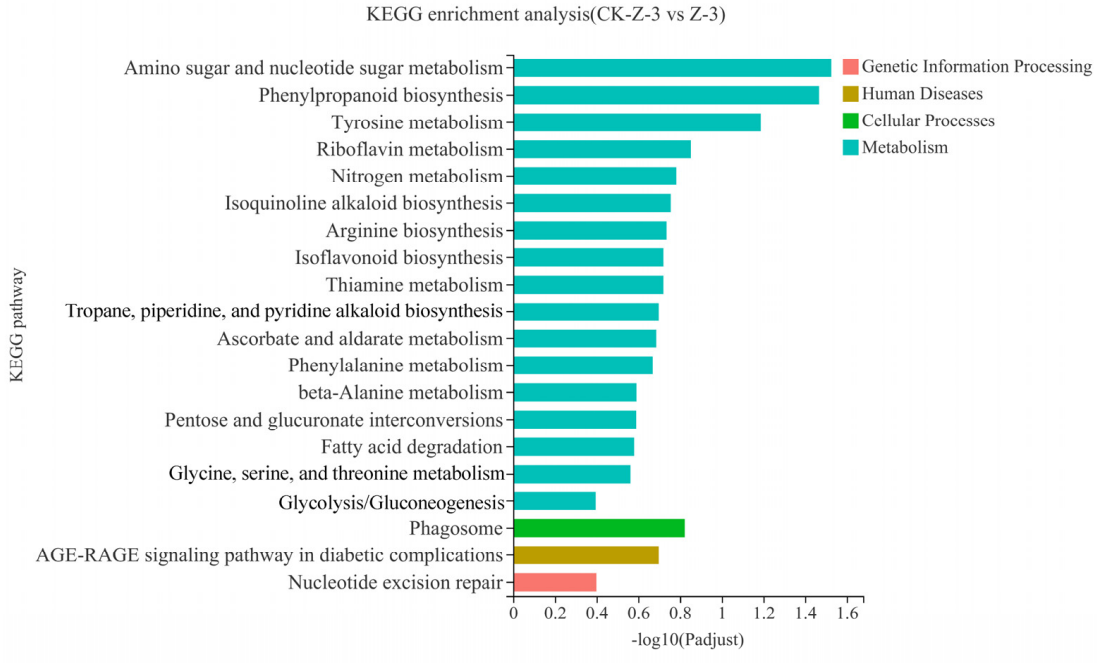
A total of 51,785 unigenes were annotated against Nr, Nt, Swiss-Prot, Pfam, COG, GO, and KEGG databases using BLASTx (E-value < 1×10^{-5}). Among them, 43,000 (44.39%), 18,441 (19.04%), 36,250 (37.42%), 50,576 (52.21%), 30,749 (31.74%), 31,337 (32.35%), and 51,785 (53.46%) unigenes were annotated to the Nr, Nt, Swiss-Prot, Pfam, COG, GO, and KEGG databases, respectively (Supplementary Figure S3).

3.4. Screening of DEGs

Using the Nr annotation results, GO functional annotations of unigenes were analyzed using Blast2GO software, while WEGO software was employed for the functional classification of unigenes. A total of 1755 significant Differentially Expressed Genes (DEGs) were assigned to one or more ontologies based on the criteria of $|\log2FC| > 1$ and False Discovery Rate (FDR) < 0.05: 44 unigenes were upregulated and the other 41 unigenes were downregulated (CK-Z-3 vs. Z-3), 904 unigenes were upregulated and the other 182 unigenes were downregulated (CK-Z-6 vs. Z-6), and 558 unigenes were upregulated and the other 26 unigenes were downregulated (CK-Z-9 vs. Z-9). The significant DEGs were used for the subsequent analysis (Supplementary Figure S4).

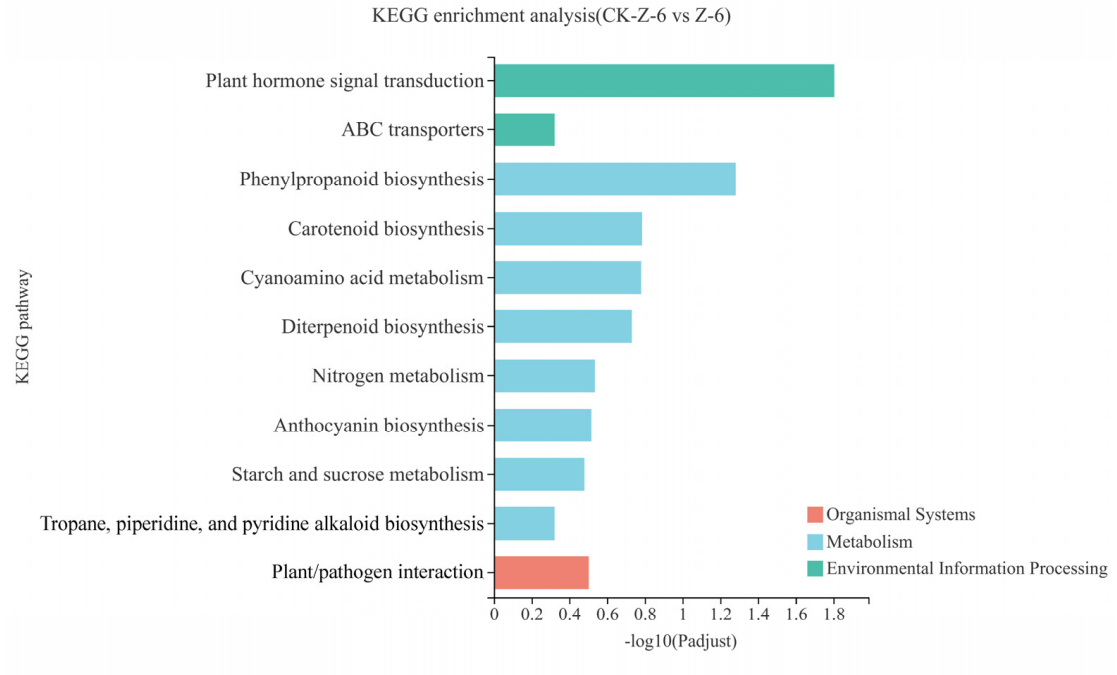
3.5. KEGG Pathway Enrichment Analysis

Annotation analysis of the KEGG pathway was performed on the differential genes of the three comparison combinations. DEGs of CK-Z-3 vs. Z-3 were significantly enriched in amino sugar and nucleotide sugar metabolism, Phenylpropanoid biosynthesis, and Tyrosine metabolism; DEGs of CK-Z-6 vs. Z-6 were significantly enriched in Phenylpropanoid biosynthesis and plant hormone signal transduction; DEGs of CK-Z-9 vs. Z-9 were significantly enriched in nitrogen metabolism, sulfur metabolism, taurine and hypotaurine metabolism, linoleic acid metabolism, cysteine and methionine metabolism, alanine, aspartate and glutamate metabolism, arginine biosynthesis, glycosaminoglycan degradation, and the MAPK signaling pathway—plant (Figure 2).

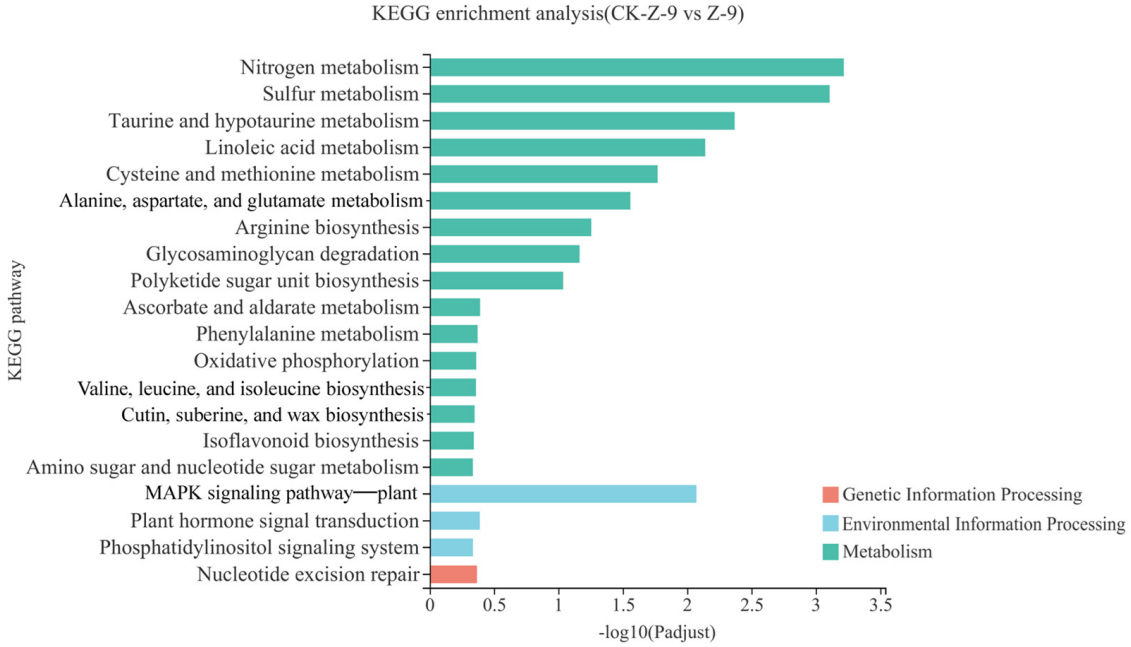


(**a**)

Figure 2. *Cont.*



(**b**)



(**c**)

Figure 2. Classification map of differentially expressed genes for KEGG enrichment analysis in each comparison combination: (**a**) differential gene annotation after 3 days of inoculation with rhizobia versus 3 days of non-inoculation treatment; (**b**) differential gene annotation after 6 days of inoculation with rhizobia versus 6 days of non-inoculation treatment; (**c**) differential gene annotation after 9 days of inoculation with rhizobia versus 9 days of non-inoculation treatment.

Note: The vertical coordinate indicates the KEGG pathway, and the horizontal coordinate indicates the significance level of enrichment, corresponding to the height of the column, where the smaller the FDR and the larger the −log10 (FDR) value, the more significantly enriched the KEGG pathway is.

3.6. Interpretation of Nitrogen Metabolism Pathway

The biological process of the nitrogen cycle is a complex interplay among many microorganisms catalyzing different reactions, where nitrogen is found in various oxidation states ranging from +5 in nitrate to −3 in ammonia. The DEGs of CK-Z-3 vs. Z-3 were searched in the KEGG database, and only one gene (K02575) with a KO number obtained in the KEGG database was screened in the nitrogen metabolism pathway, enriched in the Nitrate/nitrite Transporter (Nrt), in the early stage of the pathway. The DEGs of CK-Z-6 vs. Z-6 were searched in the KEGG database, and a total of four genes with KO numbers in the database were screened: K01674, K01673, K10534, and K00366, which are enriched in the nitrogen metabolism pathway in the carbonic anhydrase (4.2.1.1), which is downstream of the pathway, and in the Nitrate Reductase (NR) and ferredoxin-nitrite reductase (1.7.7.1) downstream of the pathway; the CK-Z-9 vs. Z-9 DEGs in the nitrogen metabolic pathway were similarly detected as K01674 and K01673 enriched in carbonic anhydrase (4.2.1.1). In addition, K01915, enriched in glutamine synthetase (6.3.1.2), and K00261, enriched in glutamate dehydrogenase (1.4.1.3), both downstream of this pathway, were detected (Figure 3).

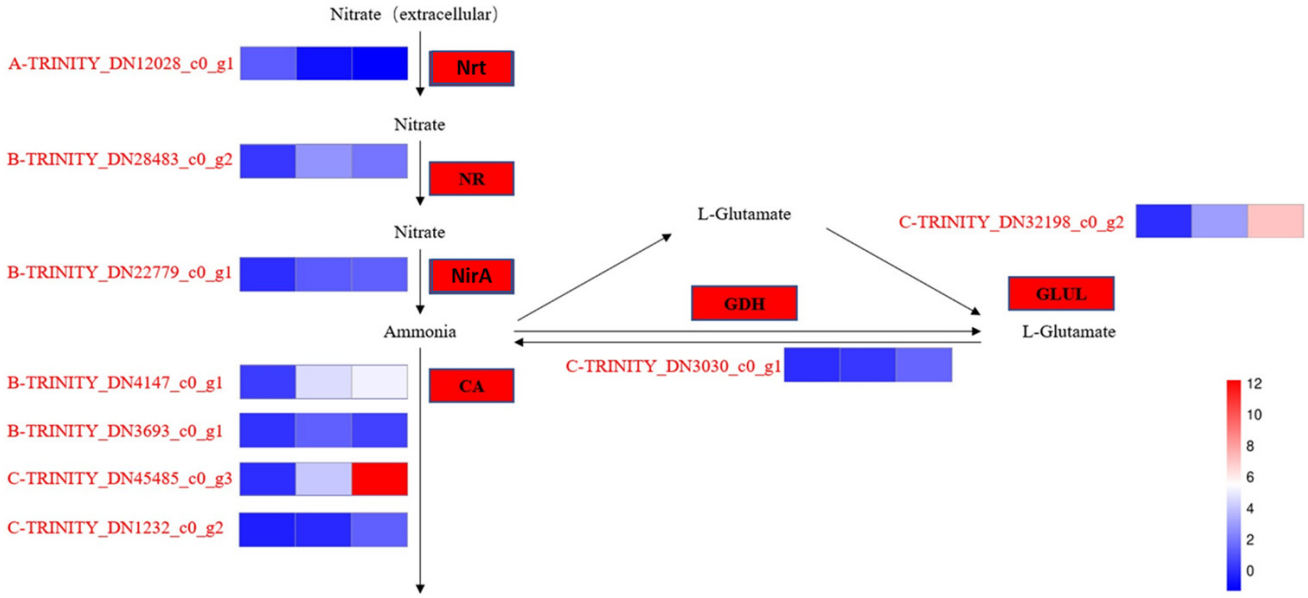


Figure 3. Differential gene expression profiles of nitrogen metabolism pathway under different treatments.

Note: A-gene serial number represents DEGs under CK-Z-3 vs. Z-3 treatment, B-gene serial number represents DEGs under CK-Z-6 vs. Z-6 treatment, C-gene serial number represents DEGs under CK-Z-9 vs. Z-9 treatment. Red indicates upregulated DEGs and blue indicates downregulated DEGs. Nrt: Nitrate/nitrite Transporter; NR: Nitrate Reductase; NirA: Ferredoxin-Nitrite Reductase; CA: Carbonic Anhydrase; GDH: Glutamate Dehydrogenase (NAD(P)+); GLUL: Glutamine Synthetase.

3.7. Interpretation of Flavonoid Synthesis Pathway

Flavonoids play vital roles during different plant/microbe symbioses, such as legume/rhizobia symbiosis, AM symbiosis, and actinorhizal symbiosis [20,21]. In legume/rhizobia symbiosis, it is well established that flavonoids play an integral role in the initial signal exchange between the two symbiotic partners [22]. The DEGs of CK-Z-3 vs. Z-3 were searched in the KEGG database and no differential genes were screened in the flavonoid synthesis pathway. Two differential genes (K00487 and K05280) were screened in the flavonoid synthesis pathway 6 days after inoculation. Among them, K00487

is enriched in trans-cinnamic acid 4-monooxygenase (1.14.14.91), which is located upstream of this pathway and upregulated in the Z-6 treatment, and is involved in the production of p-coumaroyl-CoA. K05280 was enriched in flavonoid 3′-monooxygenase (1.14.14.82), which is involved in the synthesis of compounds such as Butein, yellow pigment, eriodictyol, rehmannioflavone, xanthophyll, and quercetin, all of which were upregulated in the Z-6 treatment and located downstream of the flavonoid synthesis pathway (Figure 4).

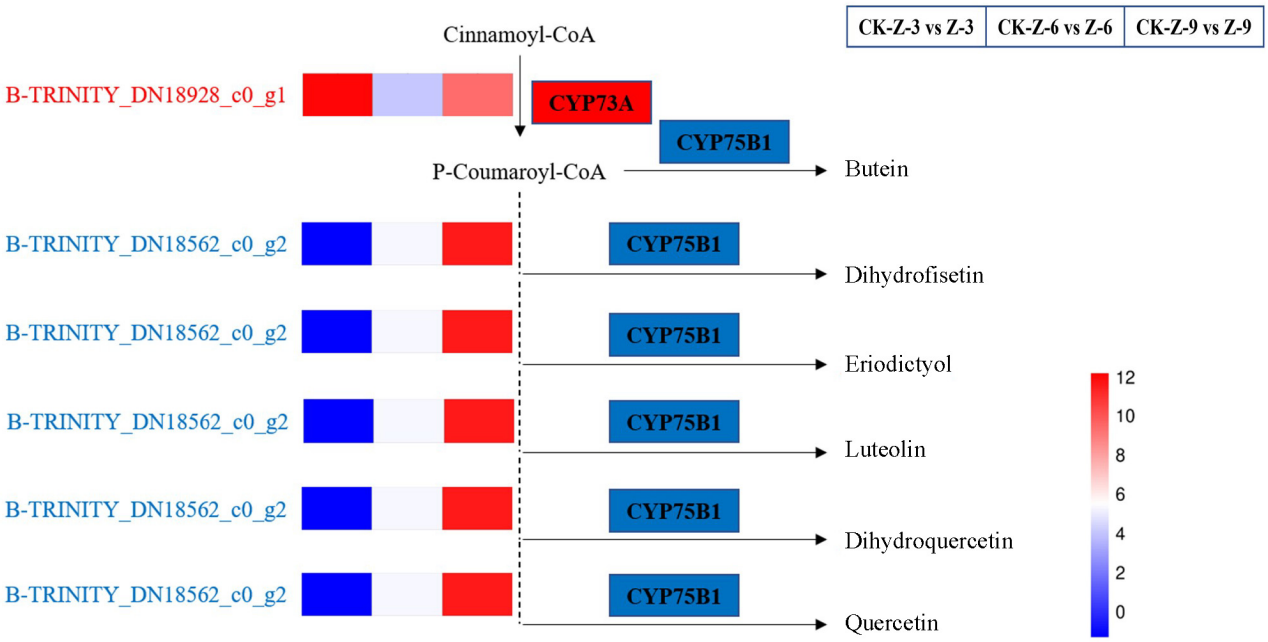


Figure 4. Differential gene expression profiles of flavonoid biosynthesis and metabolism pathway under different treatments.

Note: B-gene serial numbers represent DEGs under CK-Z-6 vs. Z-6 treatment. CYP73A: trans-cinnamate 4-monooxygenase; CYP75B1: flavonoid 3′-monooxygenase.

3.8. Weighted Gene Co-Expression Network Analysis (WGCNA) and Gene Set Enrichment Analysis (GSEA)

Another key finding of this study is the identification of tissue-specific gene modules through Weighted Gene Co-expression Network Analysis (WGCNA) (Figure 5). The objective of WGCNA was to identify co-expressed gene modules and to explore the core genes within these modules. A hierarchical clustering tree was constructed by calculating the correlation coefficients between all gene pairs [23]. Different branches of the clustering tree correspond to distinct gene modules, with different colors representing each module. Based on the weighted correlation coefficients of the genes, genes were classified according to their expression patterns, and those exhibiting similar patterns were grouped into a single module. The identification of hub genes within these modules offers valuable insights into the molecular mechanisms underpinning nodule nitrogen fixation in Clover. Here, seven co-expression modules were identified by WGCNA, among which the "red" and "green" modules were significantly associated with not being inoculated with rhizobia treatment. Analysis of the module relationships suggested that the "red" module, containing 32 genes, was highly positively correlated with levels of treatment of uninoculated rhizobia after 6 days (r = 0.646, p = 0.00378). The abundance of inoculation of rhizobia for 3 days of treatment was highly negatively correlated with gene expression in the "grey" and "green" modules, containing 73 and 37 genes, both with a coefficient of −0.646 (p = 0.00378). The abundance of uninoculated rhizobia treatments for 6 and 9 days was positively correlated

with gene expression in the "yellow" module, containing 158 genes, with coefficients of 0.474 (p = 0.0469) and 0.503 (p = 0.0334), respectively. These results suggested that the genes in this module were not associated with nodulation and nitrogen fixation in the plants. The abundance at 6 and 9 days of inoculation with rhizobia treatment was positively correlated with the expression of genes in the "turquoise" module, containing 851 genes, with coefficients of 0.474 (p = 0.0469) and 0.417 (p = 0.0851), respectively (Figure 5a). These results indicated that the genes in this module were most likely involved in regulating nodulation and nitrogen fixation processes in plants. To verify this idea, KEGG enrichment analysis of genes in the "turquoise" module revealed that genes in this module were significantly enriched in nitrogen metabolism, phytohormone signaling, and plant/pathogen interactions, further suggesting that genes in this module are involved in nodulation and nitrogen fixation processes in plants (Figure 5c). Finally, the 30 hub genes of the module were obtained by visualization network analysis (Figure 5d).

KEGG enrichment analysis was performed on all gene sets in the metabolic pathway for both sets of samples using the GSEA method. The gene set was significantly enriched in the Phenylpropanoid biosynthesis pathway (MAP00940) and was significantly upregulated in the treatment inoculated with rhizobia and downregulated in the control. In the GSEA analysis, the leader subunit contained a total of 16 genes, and the heat map of the leader subset showed that only one gene (gene id: *TRINITY_DN7551_c0_g1*) was significantly upregulated in the treatment inoculated with rhizobia, while it was downregulated in the control and had the highest ranking metric score, thus indicating that this gene played a role in regulating nodule nitrogen fixation in the plants (Supplementary Table S2).

3.9. qRT-PCR Identification of DEGs

To validate the consistency of the RNA-seq data, ten differentially expressed genes associated with key metabolic pathways were randomly selected from each of the three comparison combinations and analyzed using quantitative Real-Time PCR (qRT-PCR). The results, presented in Figure 6, demonstrate a strong correlation between the qRT-PCR data and the RNA-seq expression profiles, confirming the accuracy of the transcriptome sequencing data.

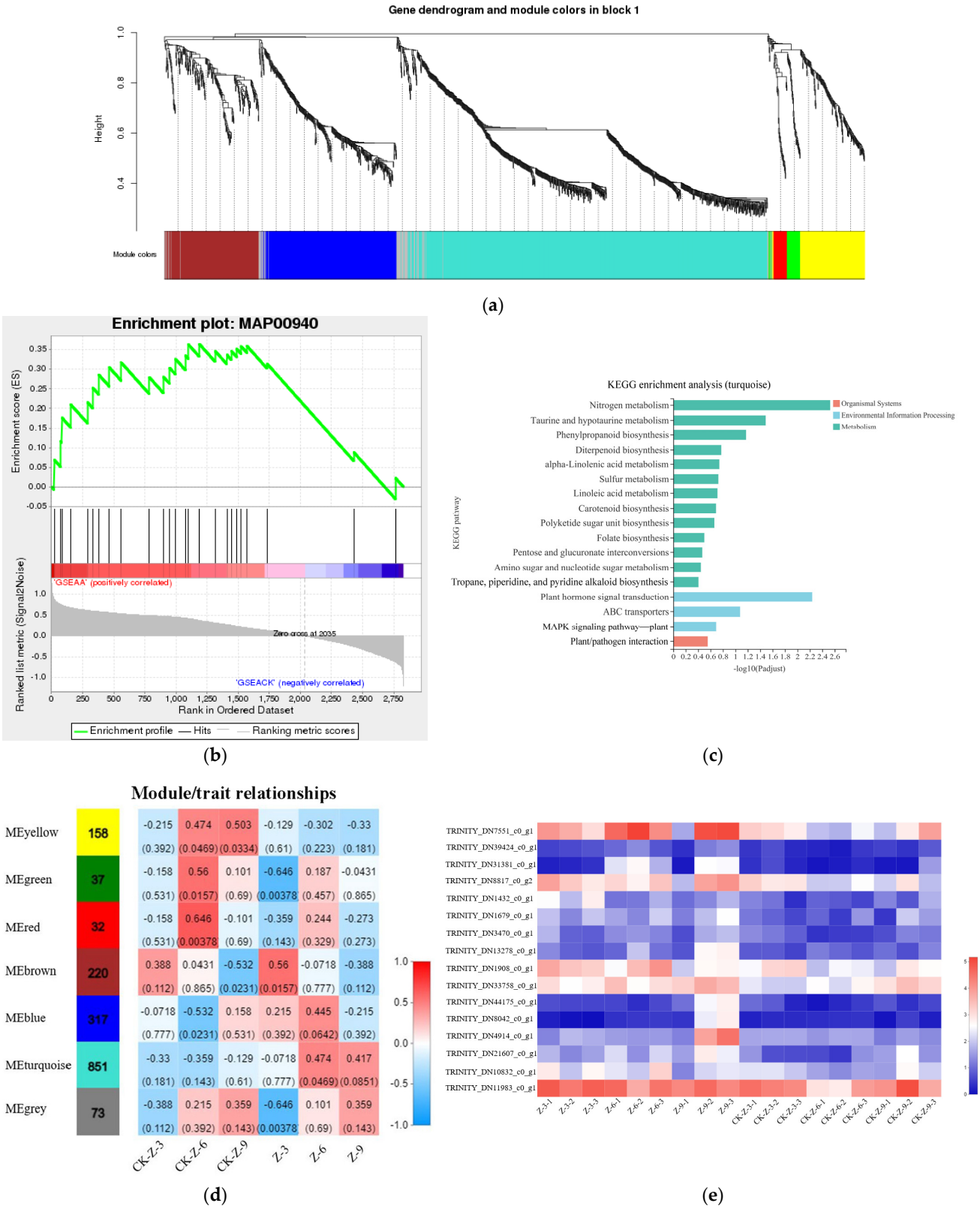


Figure 5. Weighted Gene Co-expression Network Analysis (WGCNA) between inoculated rhizobia treatment and control treatment: (**a**) hierarchical clustering showing 7 modules of co-expressed genes. Each leaf in the tree represents one gene; (**b**) correlation of modules with each treatment group and the corresponding *p*-values. The left panel shows the 7 modules and the number of genes in each module. The right panel shows a color scale for module/trait correlations from −1 to 1; (**c**) KEGG enrichment analysis of "turquoise" module; (**d**) GSEA analysis showing enrichment; (**e**) leading subset heat map.

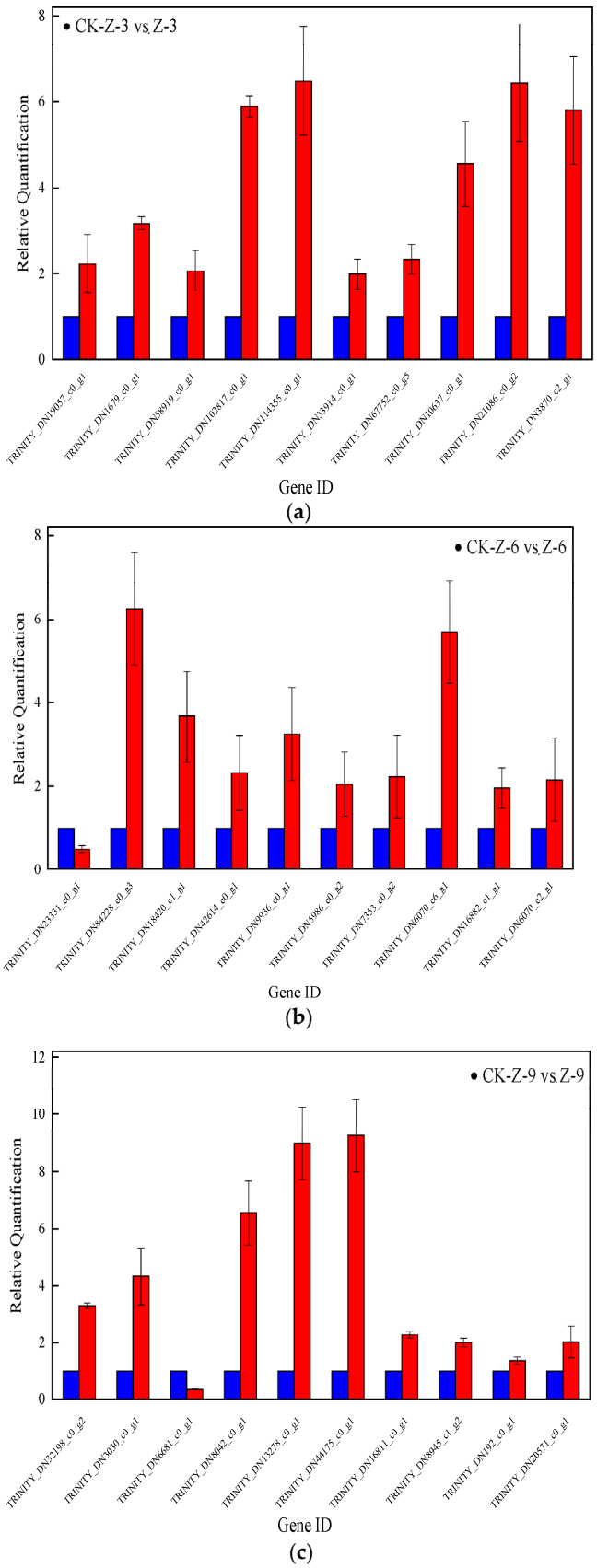


Figure 6. qRT-PCR verification of differentially expressed genes. (**a**) qRT-PCR results of the first comparison group; (**b**) qRT-PCR results of the second comparison group; (**c**) qRT-PCR results of the third comparison group.

4. Discussion

Legumes can form a special symbiosis with rhizobia, thereby fixing atmospheric N_2 for use by the ecosystem. The rational and effective use of legumes for symbiotic nitrogen fixation not only reduces the environmental impact of hemoglobin derivatives but also helps to mitigate soil eutrophication, soil acidification, and groundwater table contamination. Therefore, it is particularly important to carry out research on the molecular mechanism of nitrogen fixation by legume nodules.

In this study, five rhizobia strains were obtained by screening, isolating, and purifying the rhizobia of "Mengnong clover No. 1", and these strains were inoculated with the progeny of Caucasian clover and white clover for subsequent studies.

4.1. Effect of Rhizobia Inoculation on Plant Growth and Nodulation

Inoculation of legume crops with nitrogen-fixing soil bacteria, collectively known as rhizobia, has been widely used to improve legume productivity in fields [24]. Numerous studies have shown that legume forage can effectively improve forage yield and quality by inoculation with rhizobia, and plants inoculated with rhizobia can produce effective root nodules, thus improving crop growth performance, promoting root development, and increasing the number of root nodules and fresh dry weight of root nodules per plant [25].

Inoculation with an *R. gallicum* strain induced growth of bacterial communities that have often been reported as PGPM (plant growth-promoting microorganisms) [24]. The extent of these changes was also seen in the next rotation crop. Therefore, rhizobial inoculation resulted in increased shoot growth, number of pods, and grain yield of faba bean [26] and lentil [27] compared with non-inoculated controls. In our study, the plant height of clover plants inoculated with rhizobia significantly increased compared with CK, with the best effect being the treatment inoculated with the No. 5 strain. This may be because a strict indoor control trial was conducted in this experiment and a nitrogen-deficient nutrient solution was chosen for the plants, resulting in different results. In addition, substances secreted into the rhizosphere by rhizobia, including acetoin, 2,3-butanediol, siderophores, auxins, and other phytohormones, have plant growth-promoting properties [28].

Inoculation with rhizobia significantly increased the above-/below-ground biomass of clover plants. One reason could be that at the beginning of rhizobia inoculation, when the plants had not yet developed nodulation, a nitrogen-deficient nutrient solution was applied to the plants, resulting in a lack of nutrients for the plants, while with the appearance of root nodules, the N_2 in the air was fixed by them, providing nutrients for the plants, which allowed them to grow rapidly, increasing plant biomass. On the other hand, after inoculation with rhizobia, the plant provides more loci for rhizobia by promoting root growth and development, increasing the plant's below-ground biomass. This is similar to the findings of many research scholars.

After inoculation with rhizobia, legumes form root nodules with nitrogen-fixing capacity through specific recognition and interaction to improve the growth performance of legumes, promote root development, and increase the number of root nodules per plant and the fresh dry weight of root nodules, similar to other observations [25,29–31]. In this experiment, the material chosen was the progeny of a cross between Caucasian clover and white clover, and previous studies have shown that Caucasian clover is highly specific for the recognition of rhizobia. Researchers have crossed Caucasian clover with white clover in an attempt to obtain a clover variety that combines the best traits of both, but the results were not satisfactory. The inoculation of rhizobia isolated from Mengnong clover No. 1 resulted in nodulation of the hybrid progeny, which was similar to the results of Mitchell's study [2]. During the experiment, the vermiculite selected was completely sterilized and

only played the role of fixing and supporting the plants. The plants in each group of treatment were placed in isolation to avoid the mutual influence between the strains, and the nitrogen-deficient nutrient solution was selected so that the nitrogen nutrients for the plants could only be obtained by nitrogen fixation in the root nodules during the growth process. In this study, all five selected rhizobia strains were able to symbiose with the test material for nodule fixation, but there were differences in nitrogen fixation, nodule capacity, and plant growth promotion. Among them, the inoculation effect of No. 5 was the best, and the phenotype, root system, and rhizome index of the plants were significantly higher than the other treatments, but its nitrogen-fixing enzyme activity was slightly lower than that of the No. 3 strain, which was initially screened as the best matching strain for this clover. The authors of [32] conducted a trial on white clover inoculated with native rhizobacteria (CHB1120, CHB1121) and showed that inoculation with rhizobia significantly increased the plant's root nodule number, effective root nodule number, and nitrogen-fixing enzyme activity. In the rhizosphere of alfalfa, rhizobial inoculation significantly increased microbial biomass, thereby enhancing crop yields. Moreover, intercropping promoted better plant growth, which, in turn, improved the quality and quantity of root exudates and facilitated the turnover of root biomass into the soil. These processes subsequently supported microbial growth within the rhizosphere [33,34]. Additionally, rhizobial inoculation has been shown to induce positive changes in soil structure and microbial communities. One key characteristic of plant-associated bacteria is their ability to produce phytohormones, which stimulate an increase in the size of the plant root system. This, in turn, allows for the exploitation of a larger soil volume, thereby enhancing mineral and aqueous nutrient availability to the plant and promoting overall plant growth and development [35]. The authors of [27] found that the inoculation of peanut plants with exogenous rhizobia significantly promoted the growth (nodule number, peg number, shoot fresh weight, and effective peg rate) of host plants during different developmental stages. The authors of [36] also conducted a rhizobia inoculation experiment with common bean plants, and from the 36 tested rhizobia isolates, the best results were obtained with isolate H20 (Rhizobium tropici), which presented an REI of around 180%, using SEMIA 4077 as the reference strain. In our study, similar conclusions were reached, possibly due to the fact that inoculation of rhizobia might have profound impacts on the host plant growth via the regulation of complex interaction networks of rhizobacteria. On the other hand, it might be due to the fact that clover adjusts its symbiotic capacity to its N demand by modulating C allocation for the expansion of nodules, which in turn promotes plant growth [37].

4.2. Transcriptomic Study of Inoculated Rhizobia

The advances in next-generation sequencing have led to a burst of transcriptomic studies investigating host gene expression changes during plant/rhizobia interactions [38]. In this study, after sequencing the transcriptome of a total of 18 samples of clover roots after 3, 6, and 9 days of rhizobia inoculation, a total of 114.72 Gb of Clean Data was obtained, and each sample of Clean Data reached more than 6.06 Gb, yielding a total of 97,066 unigenes. By differential gene expression analysis, the DEGs generated at all time points had more downregulated genes than upregulated genes, and 44 upregulated genes and 41 downregulated genes were identified in the comparison combination of CK-Z-3 vs. Z-3. In total, 904 upregulated genes and 182 downregulated genes were identified in the comparison combination of CK-Z-6 vs. Z-6, whereas 558 upregulated genes and 26 downregulated genes were identified in the comparison combination of CK-Z-9 vs. Z-9. The number of DEGs produced at 3, 6, and 9 days of inoculation with rhizobia was increasing and then decreasing, and the number of upregulated genes was more than the number of downregulated genes in all cases, indicating that the primary defense response was

triggered in the early stage of infestation (3 days), and with further infestation by rhizobia, it caused the plants to develop a more advanced defense system to resist rhizobia, which induced the expression of a large number of defense-related genes, and the appearance of the middle stage of infestation (6 days). A large increase in the number of differential genes was observed. In contrast, at the late stage of infestation (9 days), the primary defense response of plants gradually stabilized, leading to a decrease in DEGs.

In the comparative combination of CK-Z-3 vs. Z-3, DEGs were significantly enriched in amino acid sugar and nucleotide sugar metabolism, with one upregulated gene and two downregulated genes; Phenylpropanoid biosynthesis, with three upregulated genes and one downregulated gene; and tyrosine metabolism, with two upregulated genes. It can be seen that the plants released flavonoid compounds to the root zone and that flavonoids contributed significantly to the adaptation of the plants to changing environmental conditions [39,40]. Phenylpropanoid biosynthesis has a strongly inducible synthetic pathway and is particularly sensitive to bacterial stimuli [41]. In addition to flavonoid compounds, amino acids in root secretions can also promote colonization by rhizobia. During the symbiosis between rhizobia and clover, the rhizobia became nutritionally deficient due to the cessation of amino acid synthesis in the bacteroid, and the plant supplied amino acids to the bacteroid as a nitrogen source before symbiotic nitrogen fixation [42].

In the comparative combination of CK-Z-6 vs. Z-6, DEGs were significantly enriched in plant hormone signal transduction, with 15 upregulated genes and 4 downregulated genes, and phenylpropanoid biosynthesis, with 16 upregulated genes and 1 downregulated gene. Synergistic expression of anthocyanins, lignans, and terpenoids in contrasting combinations was found in the transcriptome dataset of this study. Some researchers found that lignin modification increases the number of root nodules, but negatively affects root development and has limited potential to promote nitrogen fixation activity [43]. Phenylpropanoids act as low molecular weight antibiotics, anthelmintic, or signaling molecules in plant/microbe interactions and are involved in plant defense responses [44].

In the contrasting combination of CK-Z-9 vs. Z-9, significant enrichment was observed in nitrogen metabolism, sulfur metabolism, taurine and hypotaurine metabolism, linoleic acid metabolism, MAPK signaling pathway—plant, cysteine and methionine metabolism, alanine, aspartate and glutamate metabolism, arginine biosynthesis, and glycosaminoglycan degradation. Asparagine is the main form of nitrogen export in the root nodule, and after amino acid is deprived, asparagine synthetase (AsnB) is induced and catalyzes the interconversion of asparagine and glutamate to aspartate and glutamine [45]. Cysteine and methionine are sulfur-containing amino acids. Cysteine is metabolized to pyruvate by several pathways. Methionine is an essential amino acid for animals, but it cannot be synthesized in animals; in bacteria and plants, methionine is synthesized from aspartic acid. Nitrogen fixation in bacteroids is activated by the metabolism of dicarboxylic acids (mainly malate and succinate). To provide the energy and reducing agent required for nitrogen fixation, these dicarboxylic acids imported into the bacteroid need to be oxidatively decarboxylated, and dicarboxylic acid oxidation requires the synthesis of acetyl CoA [46], for which pyruvate is essential.

In addition, the detection of a cation channel (*DMI1/POLLUX* and *CASTOR* (a Ca^{2+} channel)) and a transcription factor (*IPD3/CYCLOPS*), among others, is required. These genes have been validated to be necessary for the initial stages of biological nitrogen fixation in plants [47–50]. Some genes were also detected in CSP that are also required for the symbiosis between legumes and rhizobia [48,51].

In addition to rhizobia, several Plant Growth-Promoting Bacteria (PGPB) have been utilized, including *A. brasilense*, *H. seropedicae*, *B. subtilis*, *B. anthina*, and *P. kururiensis*, among others. Transcriptomic studies investigating PGPB-induced growth promotion often

use this phenotype to confirm the beneficial effects of the interaction on the plant. Although these studies have been conducted under varying experimental conditions (e.g., sterile vs. non-sterile environments) and with different host plants or PGPB strains, certain key gene expression trends have emerged. For example, genes involved in defense responses, hormone signaling, and nutrient transport were found to be differentially expressed in host plants during interactions with PGPB. In our study, these genes were also identified, providing a foundation for future research aimed at characterizing the host genetic pathways that regulate plant/PGPB interactions [50]. Isoflavone reductase and Phenylalanine Ammonia Lyase (PAL) differential genes were detected in our transcriptome data. These genes were confirmed to be expressed in the early stages of the legume/rhizobia symbiosis [52,53]. Receptor-Like Kinases (RLKs) play key roles in plant growth, defense responses, and plant/microbe symbioses [54,55]. In our study, RLKs (LysM) were significantly upregulated in samples inoculated with rhizobia, and it has been suggested that RLKs (LysM) play an important role in the perception of microbial signals during the legume/rhizobia symbiosis [56,57].

5. Conclusions

The nodulation and nitrogen fixation capacity of the progeny of Caucasian clover and white clover crosses inoculated with rhizobia are directly related to nitrogen metabolism, flavonoid biosynthesis, and Phenylpropanoid biosynthesis. This indicates the presence of a complex molecular network to regulate nodule nitrogen fixation in clover. Overall, our screening identified dominant rhizobial strains, and preliminary transcriptomic analysis revealed the plant response to rhizobia, although the precise molecular mechanisms should be further studied.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/nitrogen6010011/s1: Figure S1: Effect of five rhizobia strains (NO.1, NO.2, NO.3, NO.5, NO.9) on the phenotype of clover seedlings. Figure S2: The size distribution of Clover unigenes. Figure S3: Heat map of correlation between samples of each treatment group. Figure S4: Number of differentially expressed genes upregulated and downregulated in each comparison combination. Table S1: Fluorescent quantita-tive primer sequences. Table S2: GSEA analysis details table.

Author Contributions: P.Z.: conceptualization, data curation, formal analysis, investigation, methodology, and writing—original draft. S.W.: methodology. K.C.: conceptualization, funding acquisition, project administration, resources, supervision, and writing—review and editing. All authors have read and agreed to the published version of the manuscript.

Funding: The authors are thankful to the National Natural Science Foundation of China (31960353) for providing the financial support.

Data Availability Statement: The datasets generated for this study can be found in the NCBI sequence reads archive (SRA) database under BioProject No. PRJNA935687.

Acknowledgments: We are grateful to all the members of our team for helpful discussion on this project.

Conflicts 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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