

Transcriptomics-based Mining and Analysis of Low Nitrogen Tolerance Related Genes in Foxtail Millet

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Abstract

Background: Nitrogen (N) is a nutrient element crucial for the growth and development of crops. A better understanding of the effects of low-N stress on millet (*Setaria italica* L.) production, a model crop for studying low-N tolerance, and the underlying molecular mechanisms responsible for low-N stress responses is vital to improvement of agricultural production.

Results: In this study, we used RILs as materials, its parental lines 'Yugu 28,' a low N-tolerant millet variety, and 'Qiyue Huang,' a low N-sensitive variety. A transcriptomic study using leaves collected twenty-one days after normal and low-N treatment, the N concentration was 2.5 and 0.5 mmol·L⁻¹, respectively. In total, 894 differentially expressed genes (DEGs) were identified between normal and low-N treatment. Further analysis revealed Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways related to amino acid, carbon, N, and lipid metabolism and secondary metabolite biosynthesis. Co-regulation analysis suggested that 15 DEGs contributed to the formation of a regulatory network involving multiple biological processes and pathways including nitrogen or amino acid biosynthesis, uptake, metabolism and utilization. RNA-seq analysis also showed that some nitrogen metabolism-related DEGs were highly expressed in low-N stress compared to normal N conditions. The changes in expression of low N-responsive genes determined by RNA sequencing were confirmed by reverse-transcription PCR for nine genes.

Conclusions: The transcriptome is beneficial for identifying the candidate genes of millet in response to low nitrogen stress, *SiGS1* (LOC101764285) and *SiNPR2* ((LOC101764285)) were identified as potential candidate genes for improving low nitrogen stress tolerance. These results are a first step to understanding the molecular mechanisms of low-N tolerance in foxtail millet and lay a foundation for its genetic improvement.

Background

Nitrogen (N) is a mineral element essential for crop growth, which plays a vital role in maintaining normal growth, metabolism, high yield, and quality of crops. N in soil mainly exists in the form of nitrate N (NO₃⁻) and ammonium N (NH₄⁺); the form and content of N in the soil are often the key factors limiting yield and quality^[1, 2]. Therefore, using N fertilizer in agricultural production has become necessary to ensure yield and quality. However, excessive application leads to crop lodging, breeding of diseases and pests, low fertilizer utilization efficiency, soil eutrophication, and other environmental problems^[3]. The amount of N fertilizer applied in China accounts for 30% of global consumption, but 50–75% of N in the soil is not absorbed by crops^[4]. Therefore, improving the N absorption and utilization efficiency of crops has become the key to solving this series of problems^[5, 6].

Foxtail millet (*Setaria italica* L.) is an annual gramineous crop domesticated from *Setaria* a long time ago and has a cultivation history of more than 10,000 years^[7]. Because of its strong adaptability and good mineral nutrient absorption characteristics, it is often cultivated in areas with poor soil and has gradually developed into an essential multigrain crop in China. Therefore, studying the aspects of mineral nutrient absorption and exploring the genes critical for N absorption in foxtail millet can provide a specific theoretical basis and gene resources for the genetic improvement of crop N efficiency.

When plants are subjected to low N-induced stress, their growth and development are inhibited, including stunted growth, short plants, yellow leaf color, premature aging, and enhanced root-shoot ratio. Under insufficient N supply, N absorption from the soil increased in maize plants by enhancing the root length and improving the root–shoot ratio^[8, 3, 9]. Maize preferentially distributes N and sucrose to the underground parts, promoting the growth and development of roots and thus enhancing N absorption^[10]. Compared with adequate N supply, millet seedlings under low N-induced stress mainly showed lesser plant height and leaf number, length, width, and area^[11]. The chlorophyll content of millet leaves markedly reduced when the N supply was insufficient, thus affecting the photosynthetic capacity^[12]. N uptake and transport systems in plant roots mainly include NO_3^- and NH_4^+ uptake and transport systems. *NRT1.1* was expressed in the roots, spire, and flower buds of *Arabidopsis* plants; in addition to absorbing NO_3^- from the soil, *NRT1.1* also acts as a signal receptor for NO_3^- to regulate lateral root development, seed germination, and gene expression^[13]. *NRT1.1B* regulates the microbial composition of the rhizosphere of rice roots, promotes the enrichment of N cycling-related microorganisms, establishes the framework of the main nitrate-related signal transduction pathway, plays a role in the molecular mechanisms underlying N and phosphorus balance in rice plants driven by the nitrate signal^[14, 15]. The tillering-N responsive gene *OsTCP19* was cloned. The genetic adaptation of rice to soil fertility in different parts of the world was analyzed^[16].

Foxtail millet is a self-pollinated and an important multigrain crop cultivated in north China. Due to its small genome and strong resistance to stress, drought, and barrenness, it has gradually developed into a model C_4 crop. Most studies on N-induced stress in millets have focused on the effects of agronomic traits, physiological indices, or individual genes under varying periods of low N. Therefore, by analyzing the transcriptomic differences and gene expression characteristics in millets under low and normal N, this study explored the genes for resistance to low N-induced stress in millets, laying a theoretical foundation and significance for future research concerning the cultivation of crop varieties resistant to stress.

Materials and methods

1.1 Experimental materials

Using ‘Yugu 28’ (selected by the Crop Research Institute of the Henan Academy of Agricultural Sciences, Zhengzhou, China), a low N-tolerant variety, as the maternal parent and ‘Qiyue Huang,’ a low N-sensitive early maturing variety, as the paternal parent, 120 recombinant inbred lines (RILs) were obtained through multiple generations of self-breeding.

1.2 Nitrogen stress treatment

The experiment was conducted in the intelligent greenhouse of the Institute of Grain Crops, Henan Academy of Agricultural Sciences, from July 10th to August 10th, 2022. Bright-colored and whole seeds were collected; 100 seeds of each RIL group and their parents were disinfected with 5% sodium hypochlorite solution for 10 min and rinsed with sterile water 3–5 times. They were placed in Petri dishes containing sterile filter paper, added with appropriate sterile water, and incubated at 28 °C in the dark. After five days of cultivation, 30 seeds with the same germination rate and growth status were selected and transplanted into a 1L, 96-well, blackened, dark hydroponic box; 1L of appropriate millet culture medium was added and grown in the greenhouse. The complete

culture medium was based on a modified Hogland formula (KH_2PO_4 $0.2 \text{ mmol}\cdot\text{L}^{-1}$, $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ $1.5 \text{ mmol}\cdot\text{L}^{-1}$, KCl $1.5 \text{ mmol}\cdot\text{L}^{-1}$, $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ $1.0 \text{ mmol}\cdot\text{L}^{-1}$, and NH_4NO_3 $1.25 \text{ mmol}\cdot\text{L}^{-1}$). The seedlings were grown in a greenhouse at a light/dark light cycle of 14/10 h, and the nutrient solution was changed every two days during the whole culture process. When the seedlings grew to the second leaf, the NH_4NO_3 concentration in the complete millet culture medium was used as the standard. For the normal N (NN) level, the NH_4NO_3 concentration was $1.25 \text{ mmol}\cdot\text{L}^{-1}$, and the N concentration was $2.5 \text{ mmol}\cdot\text{L}^{-1}$. For the low N (LN) level, the NH_4NO_3 concentration was $0.25 \text{ mmol}\cdot\text{L}^{-1}$, and the N concentration was $0.5 \text{ mmol}\cdot\text{L}^{-1}$. The experiment was repeated thrice.

1.3 RNA extraction, library preparation, and sequencing

The leaves of the RIL population plants treated with NN and LN levels for 21 days were extracted in three biological replicates for each line and treatment. The leaves were divided into the LN and NN groups. RNA was extracted using the polysaccharide polyphenol kit supplied by the Tiangen Biochemical Technology (Beijing) Co. Ltd. (QIAGEN, Germany). After the quality was assessed, three RNA samples obtained from the LN treatment group (L-1, L-2, and L-3) and the NN treatment group (N-1, N-2, and N-3) were mixed in equal amounts. RNA purity and integrity were ascertained by the NanoDrop 2000 spectrophotometer and the Agilent 2100/LabChip GX, respectively. The high-quality RNA samples were used to construct the cDNA sequencing libraries. Transcriptomic sequencing analysis was performed at the Beijing Baimaike Biotechnology Co. Ltd., and 6.63 GB of data was obtained. The sequencing platform used was NovaSeq 6000 (Illumina, USA).

1.4 Sequencing data analysis and gene annotation

After filtering the raw data for GC content distribution and sequencing error rates, clean reads were obtained for analysis. HISAT2 software was used to compare clean reads with millet reference genome *Setaria_italica.v2.2.genome.fa* to localize the reads precisely^[17]. The StringTie software was used to assemble the new transcripts^[18]. The annotation of functions based on sequence similarity and related metabolic pathways was performed using the GO^[19] and KEGG^[20] databases, respectively, to mine the critical genes related to low N-tolerance in millet.

1.5 Screening and analysis of DEGs

The GO and KEGG functions of DEGs were annotated by the cluster Profiler.RSEM (v1.2.12) was used to calculate the gene expression level, expressed as fragments per kilobase of transcript per million mapped reads (FPKMs). DESeq2 software was used to analyze the differences in gene expression between leaf samples collected from the plants treated with LN and NN. The screening criteria for the DEGs were: $P \text{ value} \leq 0.01$ and $|\log_2 \text{ Fold Change}| \geq 2.0$.

1.6 Chromosomal location

Chromosomal data, including the chromosome number and the position of the DEGs, were retrieved from Ensembl Plants (<http://plants.ensembl.org/index.html>). The genes were then plotted individually onto the foxtail millet chromosomes, and the resultant physical maps were displayed using the MapChart software^[21].

1.7 RT-qPCR analysis

To verify the reliability of the transcriptomic results, RNA from the LN and NN group plants were extracted and mixed according to the instructions of the Polysaccharide Polyphenol Kit (TIAGEN, Germany) supplied by the Tiangen Biochemical Technology (Beijing) Co. Ltd. Reverse transcription was performed to synthesize the cDNA using the EasyScript® first-strand cDNA Synthesis SuperMix. Among the 17 DEGs associated with low N-tolerance traits, nine were randomly selected: Seita.1G300600, Seita.3G024100, Seita.3G051900, Seita.5G052700, Seita.6G020900, Seita.9G477200, Seita.2G382100, Seita.4G124800, and Seita.8G065700 for fluorescence RT-qPCR analysis using *Actin* as the internal reference gene. The software Primer Premier 5.0 was used to design the primers, which were synthesized by the Sheng Gong Bioengineering (Shanghai) Co. Ltd. (Table 1). The relative expression of genes was calculated using $2^{-\Delta\Delta CT}$ with a threshold (Ct) standard deviation ≤ 0.5 .

Table 1 RT-qPCR primers used for analyzing the expression of genes related to low N-resistance traits in foxtail millet.	
Gene name	Primer sequence (5'–3')
<i>SiActin</i>	F:GGATACTCTTTCACCACTC
	R:GGATACTCTTTCACCACTC
<i>Seita.1G300600</i>	F:TGCTACATTTCACGACCC
	R:CTAGTGGCGGAGGTGAGAGT
<i>Seita.3G024100</i>	F:GCCGACTGCTACATTTCCTA
	R:TAGTGGCGGAGGTGAGAGTG
<i>Seita.3G051900</i>	F:GTCCTCATCCGCAATCTCT
	R:ACCCTGAGAGAACGACTGGG
<i>Seita.5G052700</i>	F:AATGGAGTTGCCCCAGTTCC
	R:GGCACAAGAGGGTACGGAAA
<i>Seita.6G020900</i>	F:TGTCCGGTGTCTGTCTTCTAC
	R:ATTGGCGTGACGGAAGTTGT
<i>Seita.9G477200</i>	F:GTTACATCTTAGTGACTCAATGCTT
	R:GATGAACCACCCTTCAGGAAC
<i>Seita.2G382100</i>	F:GTACCGCAACCCGAACATA
	R:ACAATACTCAGCGGTTCCCC
<i>Seita.4G124800</i>	F:TTCTCTGTCGTGACCGAACC
	R:GAACCGAAGGCCGAGAGAG
<i>Seita.8G065700</i>	F:CTACTCCCTCGCTCGTCCAAG
	R:TGGAGGTGTAGTAGATGACGAAG

Results and analysis

2.1 Sequencing results and data analysis

After transcriptomic sequencing of the RNA from the N-1, N-2, and N-3; and L-1, L-2, and L-3 lines was performed using the Illumina Novaseq 6000 high-throughput sequencing platform, 296 963,596 clean reads were obtained. The percentage of bases (Q20) with mass parameters not < 20 in each sample was 97.77% (N-3) and 97.93% (L-2, L-3, N-1). The percentage of bases (Q30) with quality parameters not < 30 was > 93.67% and the GC% was 55.6–56.72%. During transcriptomic sequencing, except for reverse transcription, the first few bases had a specific nucleotide preference, and the composition fluctuated; while the other positions were stable in distribution, and there was no base (AT/GC) separation (Fig. 1). All effective reads obtained by sequencing were compared with the reference genome of millet. The efficiency of comparison between the two groups of samples and the reference gene was > 91%, with the multi-site comparison rate ranging from 2.03–3.00% and the unit point comparison rate ranging from 88.65–94.12%. Furthermore, the efficiency of alignment to the exon region of the genome was 78.47% (Table 2). These results showed that the sequencing reads were high quality and suitable for further bioinformatics analysis.

Table 2
Comparison of the sample sequencing data with the reference genome and analysis.

Sample number	Chean reads	Mapped ratio	Uniquely mapped reads	Multiple mapped reads	Mapped exon region	Mapped intronic region	Mapped intergenic
N-1	51517318	96.33%	93.33%	3.00%	77.85%	18.51%	3.64%
N-2	47885170	95.86%	93.63%	2.22%	78.01%	18.38%	3.61%
N-3	44286606	95.95%	93.92%	2.03%	77.8%	18.5%	3.7%
L-1	49723490	96.27%	93.90%	2.37%	78.84%	17.75%	3.41%
L-2	56071668	91.00%	88.65%	2.35%	78.50%	17.73%	3.77%
L-3	47479344	96.45%	94.12%	2.34%	79.80%	16.63%	3.57%
Total/Average	296963596	95.31%	92.93%	2.39%	78.47%	17.92%	3.62%

2.2 Analysis and annotation of the DEGs

This study evaluated the correlation of gene expression levels among the biological repetitions (Fig. 2). The correlation coefficient $\gamma > 0.8$ among the replicates in the LN and NN groups indicated that the correlation between the samples was strong and the repeatability was excellent. DESeq2 was used to screen the DEGs in the LN and NN groups, and 894 DEGs were identified between the two samples. Compared with NN, 428 genes were up-regulated and 466 down-regulated in LN groups, respectively (Fig. 3).

2.3 GO classification and significant enrichment analysis of the DEGs

Based on the GO functional annotation, among the 894 DEGs, 744 were significantly enriched ($P < 0.05$) to biological process (BP), cell component (CC), molecular function (MF), and other three major categories of 60

functional subclasses. Through the enrichment analysis of the DEGs annotated by GO, the ten topmost highly enriched GO entries in each category were counted.

In the category of BP, the main enrichment processes were the protein translation process (GO:0006412), ribosome small subunit assembly process (GO:0000028), translation extension process (GO:0006414), chloroplast (GO:0009507), chloroplast matrix (GO: 0009570), thylakoid membrane (GO: 0009535), ribosome (GO:0005840) and cytoplasmic small ribosomal subunits (GO:0022627) (Fig. 4). In the MF category, ribosomal structural components (GO:0003735), transmembrane transporter activity (GO:0022857), Fe-S binding (GO:0051536), chlorophyll-binding (GO:0016168), and amino acid transaminase activity (GO:0004084) were the most enriched processes. The GO enrichment results revealed that the expression abundance of the aminotransferase activity in millet was high, and it could be related to its low N-tolerance of millet.

2.4 KEGG pathway enrichment analysis

KEGG pathway enrichment analysis on selected DEGs was performed to explore further the biological functions of genes associated with low N-tolerance in millet and clarify the metabolic pathways involved. Using KEGG, 637 DEGs were annotated into 106 pathways, which were classified into five categories, including metabolism, genetic information processing, environmental information processing, biological system, and cellular processes (Fig. 5). Among these, the metabolic pathways accounted for the most significant proportion, which included amino acid, carbon, glucose, and lipid metabolism and secondary metabolite biosynthesis. These results indicated that compared with genetic information, environmental information, biological systems, cellular processes, and various metabolic activities were more active under low N. Especially amino acid metabolism may be the primary metabolic pathway causing tolerance to low N in millet.

2.5 Differential expression analysis of TFs

TFs are essential in regulating N metabolism and amino acid biosynthesis in plants. A total of 441 TFs were annotated under the two N levels, which were classified into 48 families, among which 71 TFs showed marked variations in expression (Fig. 6). Additionally, 44 genes were up-, and 27 were down-regulated under low N levels. The TF family affecting N uptake and utilization in plants was C2C2-Dof, which contained four TFs; three were up-regulated, and one was down-regulated at low N levels. These three TFs, Seita.2G242400, Seita.5G052700, and Seita.9G259300, may regulate the absorption, transport, and assimilation of glutamic N.

2.6 Mining and analysis of DEGs related to low N tolerance

To further explore the genes related to regulating low N-tolerance in millet, the DEGs were screened and identified based on the published annotation of the millet genome^[22]. A total of 15 DEGs were obtained (Table 3). Comparing the expression levels under low nitrogen stress to those under normal nitrogen levels, we observed up-regulation in 10 genes, namely Seita.1G300600, Seita.2G242400, Seita.3G024100, Seita.3G051900, Seita.5G465900, Seita.5G052700, Seita.6G020900, Seita.6G078400, Seita.9G477200, and Seita.9G259300. Conversely, five genes were down-regulated, including Seita.1G186500, Seita.4G124800, Seita.5G400900, Seita.8G065700, and Seita.9G055400.

The levels of the nitrogen source regulatory protein (NRP), nitrate reductase (NR), and the aminogenic factor “Dof” were all upregulated in the pathways of N and amino acid metabolism (Table 3). In addition, the expression

levels of aspartate aminopeptidase, lysine histidine transportase-related acid permease (AAP), and glutamine synthetase (GS) encoding genes and transgenes regulating N absorption, transport, and assimilation were also upregulated. These results indicate that in the pathway of N metabolism, the synergistic activity of various enzymes can promote N absorption, transport, and assimilation, improve the N utilization rate of millet, maintain the N source balance, and sustain the normal life activities of plants.

Table 3

The differentially expressed genes associated with low nitrogen tolerance in foxtail millet under low nitrogen level.

Expression pattern	Gene ID	Gene name	Description	Log ₂ (Fold change)	Gene function
Up regulated	Seita.1G300600	LOC101766408	amino acid permease 7 isoform X2	2.03	Amino acid transmembrane transporter activity
	Seita.2G242400	LOC101765607	dof zinc finger protein MNB1A	1.45	regulation of transcription
	Seita.3G024100	LOC101764285	glutamine synthetase	30.04	glutamate-ammonia ligase activity
	Seita.3G051900	LOC101760785	nitrogen regulatory protein P-II homolog isoform	30.01	regulation of nitrogen utilization
	Seita.5G465900	LOC101755168	aspartyl aminopeptidase	1.28	metalloaminopeptidase activity
	Seita.5G052700	LOC101776499	cyclic dof factor 2	2.05	regulation of transcription
	Seita.6G020900	LOC101763560	protein NRT1/ PTR FAMILY 4.3	2.44	transmembrane transporter activity
	Seita.6G078400	LOC101766686	lysine histidine transporter 1	2.27	integral component of membrane
	Seita.9G477200	LOC101759322	protein NRT1/ PTR FAMILY 8.3 isoform X2	2.29	transmembrane transporter activity
	Seita.9G259300	LOC101771077	cyclic dof factor 1	1.77	transcription regulator activity
Down regulated	Seita.1G186500	LOC101764221	serine decarboxylase 1	-1.79	carboxy-lyase activity
	Seita.4G124800	LOC101774857	dof zinc finger protein 4	-1.25	regulation of transcription
	Seita.5G400900	LOC101786220	amino acid permease 8	-2.01	amino acid transmembrane transporter activity
	Seita.8G065700	LOC101775906	amino acid permease 3	-3.37	integral component of membrane
	Seita.9G055400	LOC101767567	tryptophan synthase alpha chain	-1.08	tryptophan synthase activity

2.7 RT-qPCR verification

To verify the reliability of the transcriptomic sequence data, nine genes under the two N-treatment levels were selected using RT-qPCR, including those encoding GS, AAP, NRP, cyclic Dof factor (CDF) TF, and NRT1/PTR. The positive correlation coefficients between RNA-seq and RT-qPCR results were high and statistically significant ($R^2 = 0.9902$, $P < 0.01$; Fig. 7), indicating that the transcriptome sequencing results were accurate and reliable.

2.8 Chromosomal location of DEGs

Regarding their chromosomal location, the 856 DEGs common to the two N levels were plotted onto nine chromosomes of foxtail millet, and the physical map was constructed, which indicated uneven distribution on the nine chromosomes (Fig. 8). For example, 104 genes were localized on chromosome 1, while chromosomes 6 and 8 possessed 47 and 49 genes, respectively. The chromosomal location analysis indicated important positional information of candidate genes related to N metabolism to develop molecular markers further.

Discussion

N plays a crucial role in many metabolic and biochemical processes in plants, directly and effectively affecting the formation of chlorophyll and assimilation through photosynthesis^[23]. The N metabolism in millet is closely related to the growth, development, and nutrient quality. Based on the low N-tolerance of millet, the mining of genes closely related to the target traits in the N metabolism pathway formed the basis for obtaining high-yield and high-quality millet. Transcriptomic sequencing can effectively mine the genes essential for controlling a certain trait in plants and obtain all information about the expression levels of the associated genes^[24]. This study used the millet genome as a reference and combined it with previous research results to conduct a comparative analysis of the genes related to low N-tolerance in millet and explore their regulatory mechanism. N metabolism, absorption, transport, and amino acid metabolism pathways related to low N-tolerance traits with more differentially annotated gene sequences selected as the basis to identify genes critical to regulating these traits.

N metabolism is an essential pathway for converting inorganic N into organic N and the biosynthesis of amino acids. After external N enters the plant, the synthesis of nitrogenous compounds is catalyzed by a series of enzymes, such as NR, to provide the nutrients required for plant growth and development^[25]. The transcriptomic data obtained in this study intuitively reflected the molecular information in millet leaves under LN to understand the related genes and the regulatory pathways involved in millet growth under LN. Compared with NN, 428 genes were up-regulated, and 466 were down-regulated at LN. GO enrichment indicated that more DEGs were enriched in the process of amino acid synthesis and metabolism at LN, and it could be inferred that low N-tolerance in millet was related to transaminase activity. KEGG analysis showed that most of the DEGs were concentrated in MP; genes related to N metabolism, amino acid (glutamic acid, aspartate, tryptophan, serine, and lysine) biosynthesis, nitrate transporters, and TFs were screened.

Under LN, the upregulated genes were mainly related to amino acid synthetase, nitrate transporter, and AAP, suggesting that an active amino acid metabolic pathway may majorly lead to low N-tolerance in millet. The glutamine synthase (GS)/glutamate synthase (GOGAT) cycle, as the focal point of the entire N metabolism, is the main pathway of crop N metabolism^[26]. The GS/GOGAT circulating enzyme system has been detected in the roots, cotyledon, leaves, and other organs of various plants. Through studying mutant plants lacking GOGAT, such as pea (*Pisum sativum*) and *Arabidopsis thaliana*, it was found that when grown in air, the glutamic acid accumulated rapidly in the leaves, while amino acid synthesis reduced. A double barley mutant (GS and Fd-GOGAT deficient) grown at high concentrations of CO₂ was exposed to air to inhibit growth, decrease CO₂

utilization, and increase NH_3 concentration in the leaves^[27]. Therefore, improving the efficiency of the GS/GOGAT cycle is an effective way to improve N use efficiency and enhance tolerance to low N-induced stress. In this study, the gene encoding GS was upregulated under LN, demonstrating that it plays a vital role in N metabolism and utilization in millet. NR is a rate-limiting enzyme in the conversion of NO_3^- to NH_4^+ (ammonia assimilation)^[28, 29]; regulating N metabolism, nitrate assimilation, and other vital metabolic pathways^[30]; and has a marked impact on the yield and quality of plants^[31]. In this study, the gene encoding NR was upregulated, indicating that an increase in its activity significantly promoted the efficiency of ammonia N assimilation in millet under low N stress, thus accelerating the absorption and utilization of N by millet and improving the tolerance of millet to low N-induced stress.

Transcription factors play a crucial role in the growth, development, and morphogenesis of plants. Among these factors, the C2C2-Dof transcription factor has shown a close association with nitrogen metabolism, as revealed from the analysis of transcriptome data. The Dof transcription factors are known to participate in various biological processes such as photoresponse, photoperiod regulation, flowering, seed development, germination, plant hormone signaling, defense response, and nitrogen metabolism^[32]. Notably, ZmDof1, identified in both Arabidopsis and rice, has been found to enhance nitrogen assimilation and promote plant growth, particularly under conditions of nitrogen deficiency^[33]. Additionally, apart from the transcription factors specifically linked to nitrogen metabolism, the discovery of ERF transcription factors, closely related to seed development^[34], has provided further insights into the genetic landscape of millet growth and development. These findings serve as valuable clues and foundations for the investigation of the nitrogen metabolism pathway in millet.

This study performed transcriptomic sequencing and functional annotation of DEGs in recombinant glutamic inbred lines under NN and LN. Through GO functional and KEGG pathway enrichment analyses, 15 genes closely related to amino acid and N metabolism and Dof TFs were screened and verified by RT-qPCR. The mechanism of their regulation during the tolerance of millet to LN was analyzed. However, further verification using overexpression and gene knockout is needed to determine the functions of the candidate genes. This study laid a foundation for the mining, cloning, and further verification of the functionally essential genes determining low N-tolerance in millet and also provided a theoretical basis for the in-depth analysis of the underlying molecular mechanism and variety improvement.

Conclusions

The transcriptome is beneficial for identifying the candidate genes of millet in response to low nitrogen stress, *SiGS1* (LOC101764285) and *SiNPR2* (LOC101764285) were identified as potential candidate genes for improving low nitrogen stress tolerance. These results are a first step to understanding the molecular mechanisms of low-N tolerance in foxtail millet and lay a foundation for its genetic improvement.

Declarations

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Competing interests The authors have no relevant financial or non-financial interests to disclose.

Author contributions All authors contributed to the study conception and design. Na Qin, Junxia Li and Zhenyan Ye designed the research. Material preparation, data collection and analysis were performed by Na Qin, Senjie Fu, Cancan Zhu and Chunyi Wang. Shutao Dai, Ya Jing and Yinghui Song provided technical assistance to Na Qin. The first draft of the manuscript was written by Na Qin, Senjie Fu conceived and supervised the writing and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data availability All data supporting the conclusions of this article are provided within the article. The transcriptome data used in the current study are available in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database (<https://www.ncbi.nlm.nih.gov/sra>) (accession number: PRJNA1052939).

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Figures

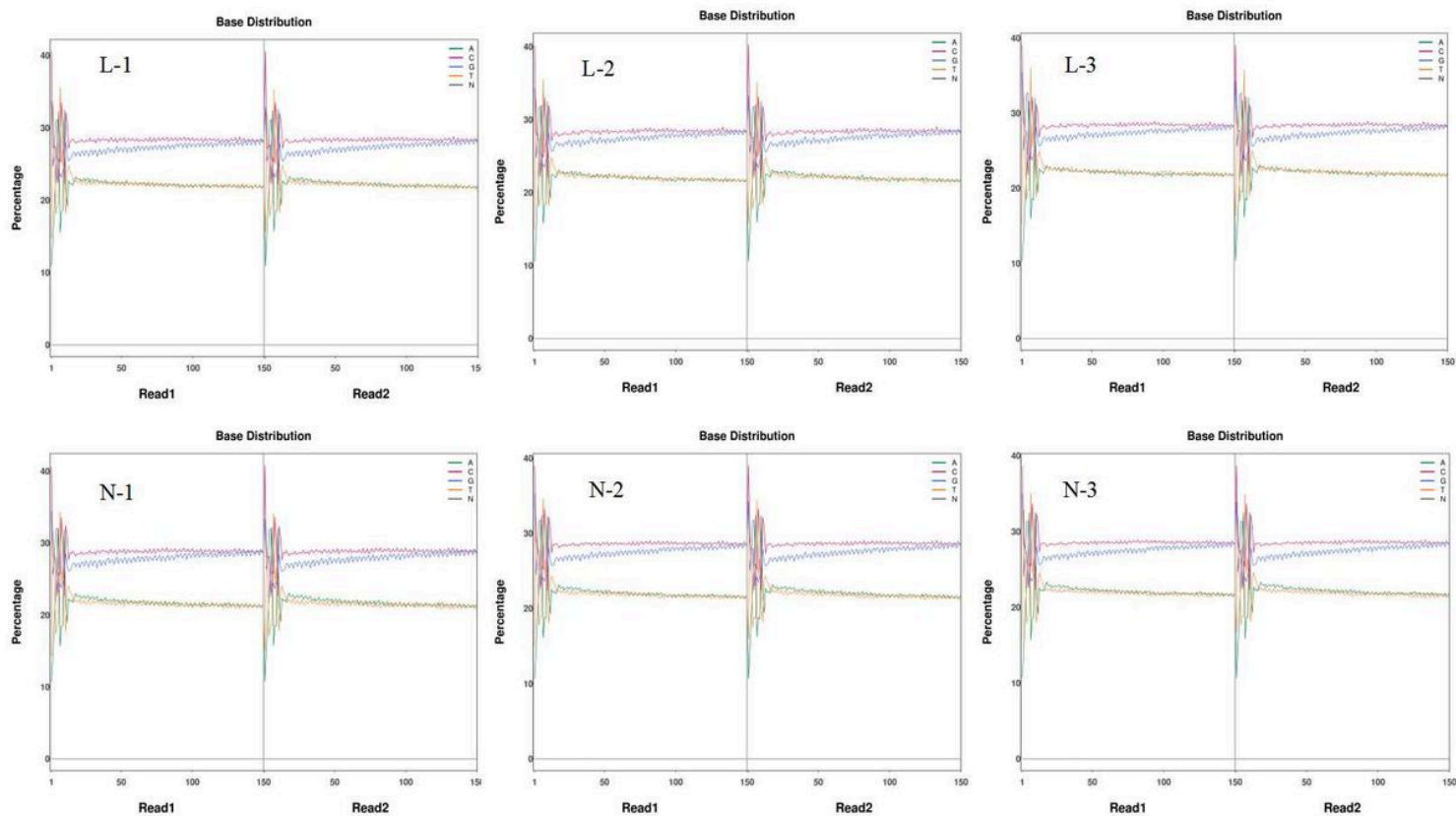


Figure 1

The composition and base distribution in RIL DNA under LN and NN levels.

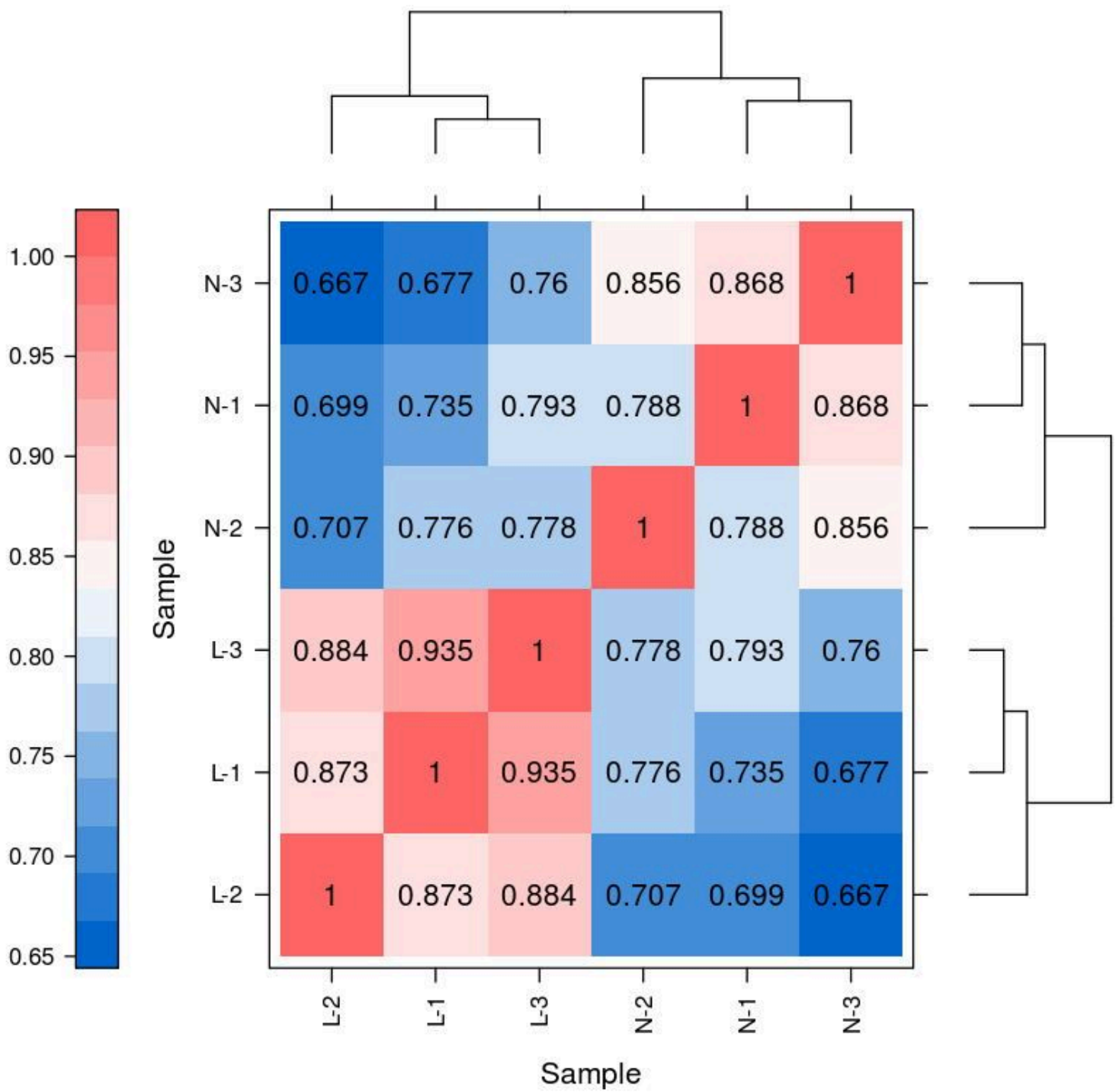


Figure 2

Correlation analysis between the normal (NN) and low (LN) nitrogen samples.

Volcano plot

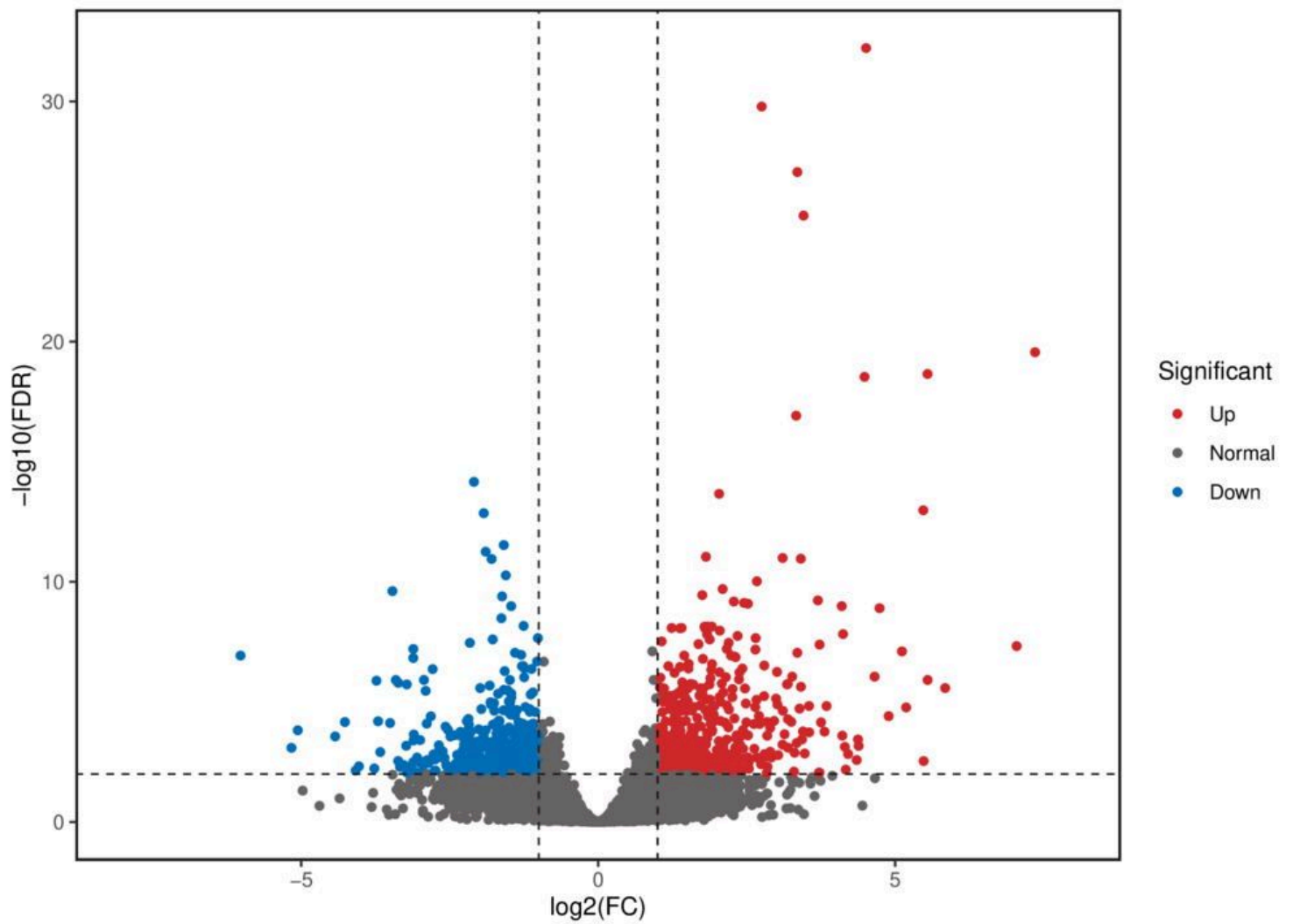


Figure 3

Volcano plot of the differentially expressed genes between the normal (NN) and low (LN) nitrogen samples.

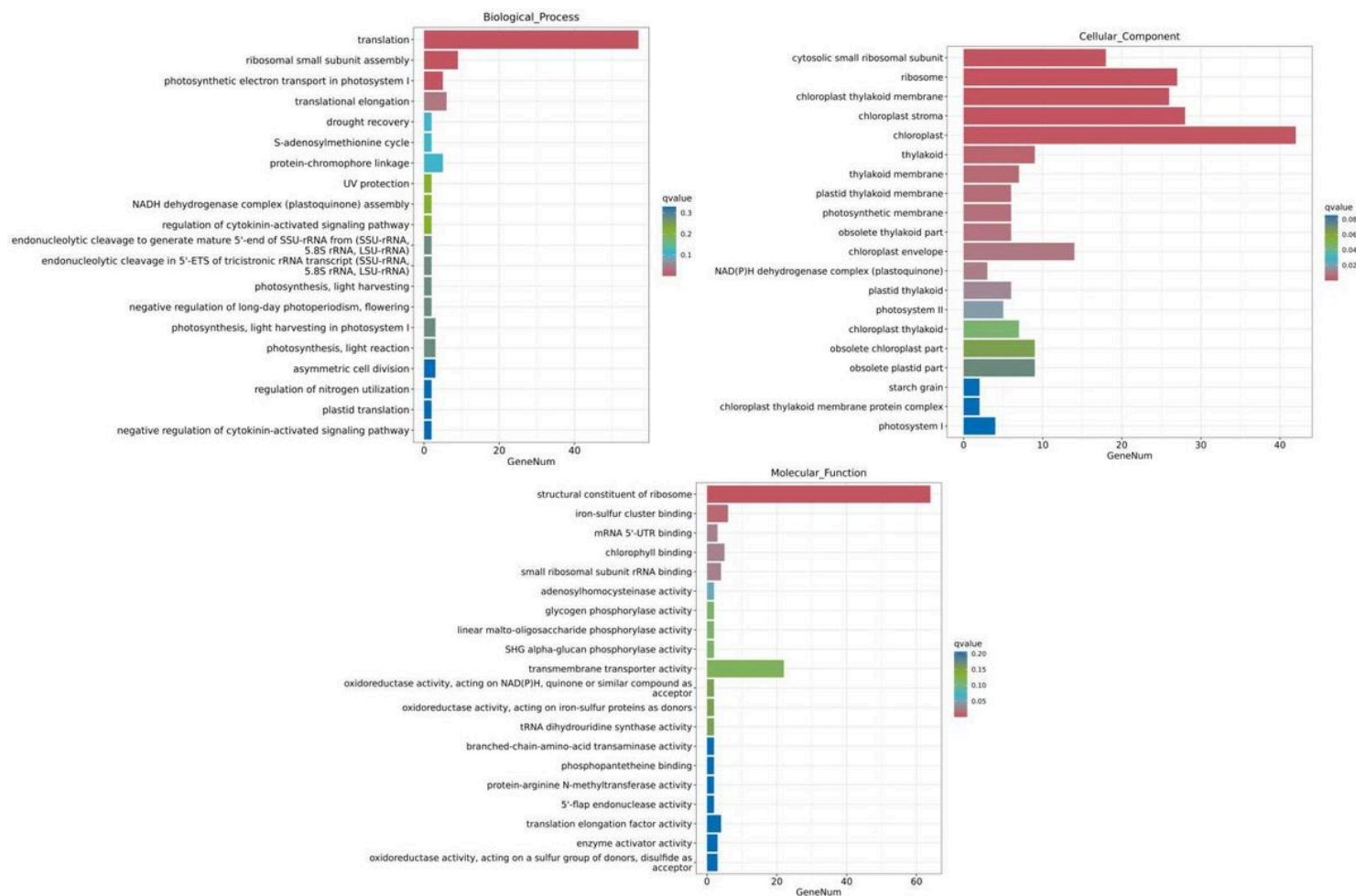


Figure 4

GO enrichment of the differentially expressed genes.

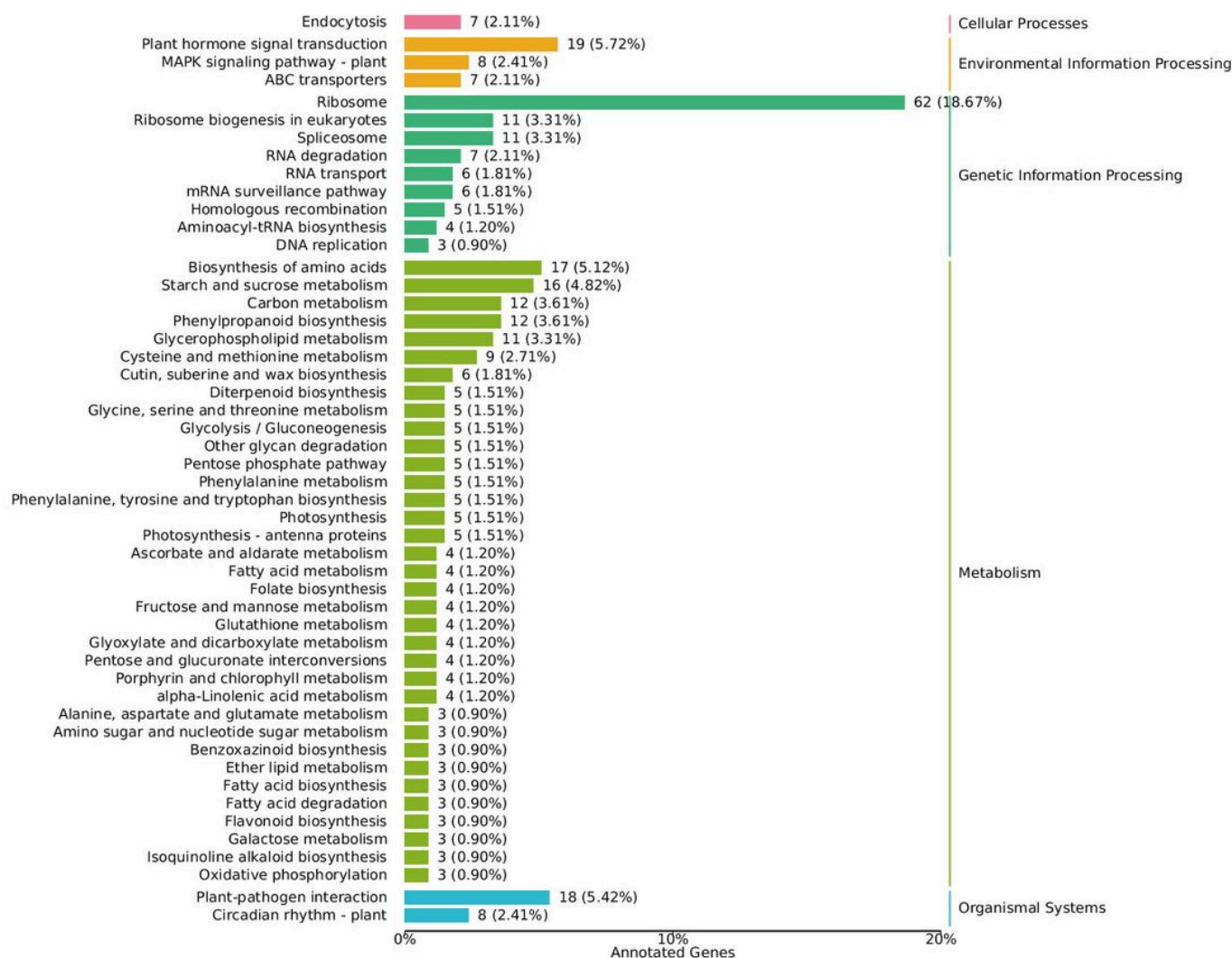


Figure 5

KEGG pathways of the differentially expressed genes identified under low and normal nitrogen levels.

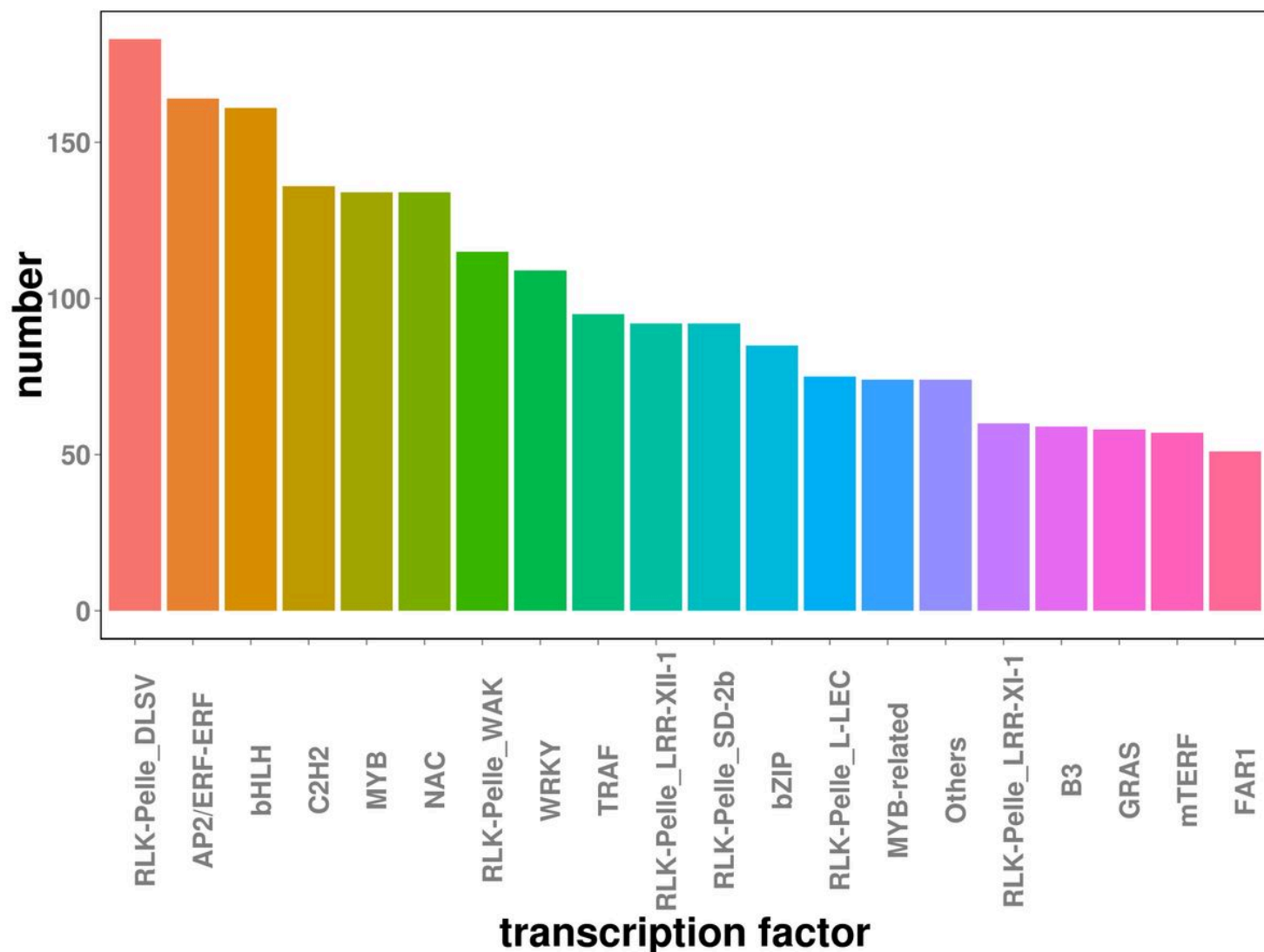


Figure 6

Transcription factor families enriched among the differentially expressed genes.

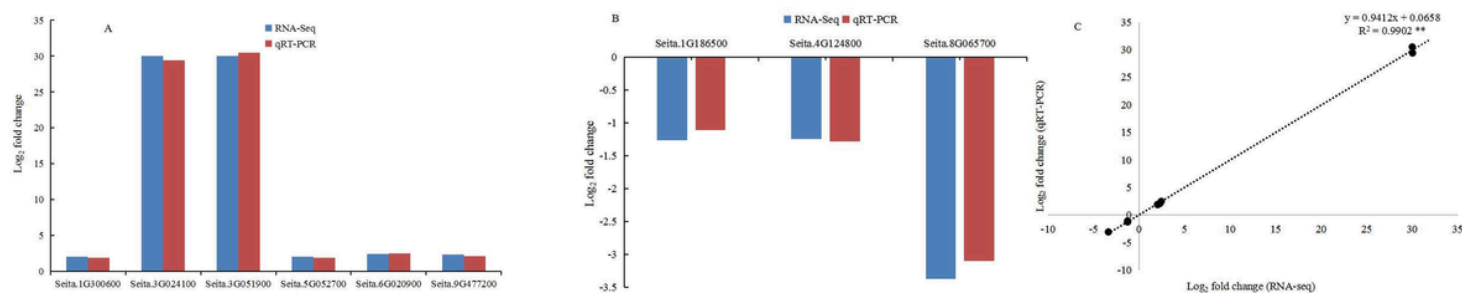


Figure 7

Correlation analysis of the RNA-seq and qRT-PCR results. (A and B) Expression levels

