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RESEARCH ARTICLE

Integrative physiological and transcriptome analysis unravels the mechanism of low nitrogen use efficiency in burley tobacco

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Abstract

Burley tobacco, a chlorophyll-deficient mutant with impaired nitrogen use efficiency (NUE), generally requires three to five times more nitrogen fertilization than flue-cured tobacco to achieve a comparable yield, which generates serious environmental pollution and negatively affects human health. Therefore, exploring the mechanisms underlying NUE is an effective measure to reduce environmental pollution and an essential direction for burley tobacco plant improvement. Physiological and genetic factors affecting tobacco NUE were identified using two tobacco genotypes with contrasting NUE in hydroponic experiments. Nitrogen use inefficient genotype (TN90) had lower nitrogen uptake and transport efficiencies, reduced leaf and root biomass, lower nitrogen assimilation and photosynthesis capacity, and lower nitrogen remobilization ability than the nitrogen use efficient genotype (K326). Transcriptomic analysis revealed that genes associated with photosynthesis, carbon fixation, and nitrogen metabolism are implicated in NUE. Three nitrate transporter genes in the leaves (*NPF2.11*, *NPF2.13*, and *NPF3.1*) and three nitrate transporter genes (*NPF6.3*, *NRT2.1*, and *NRT2.4*) in roots were down-regulated by nitrogen starvation, all of which showed lower expression in TN90 than in K326. In addition, the protein–protein interaction (PPI) network diagram identified eight key genes (*TPIP1*, *GAPB*, *HEMB*, *PGK3*, *PSBO*, *PSBP2*, *PSAG*, and *GLN2*) that may affect NUE. Less advantageous changes in nitrogen uptake, nitrogen assimilation in combination with nitrogen remobilization, and maintenance of photosynthesis in response to nitrogen deficiency led to a lower NUE in TN90. The key genes (*TPIP1*, *GAPB*, *PGK3*, *PSBO*, *PSBP2*, *PSAG*, and *GLN2*) were associated with improving photosynthesis and nitrogen metabolism in tobacco plants grown under N-deficient conditions.

KEYWORDS

burley tobacco, carbon and metabolism, N deficiency, nitrogen use efficiency, photosynthesis

1 | INTRODUCTION

Nitrogen is an essential macronutrient and key element for nucleic acids, proteins, chlorophyll, alkaloids, vitamins, and hormones and plays an essential role in plant growth and development (Meng et al., 2021). Tobacco (*Nicotiana tabacum*) usually requires large amounts of nitrogen to obtain the best yield and quality and is usually overfertilized (Sifola et al., 2018; Subsurface HWC, 2015). However, the heavy use of nitrogen fertilizers adversely affects nitrogen use efficiency (NUE), which not only leads to yield loss but also poses a threat to environmental sustainability (Wang et al., 2022). Kaiser et al. (Subsurface HWC, 2015) found that the nitrate in soil solutions from a native environment was low when compared to values in tobacco fields. Improvement in NUE will not only avert the environmental hazards caused by the application of excessive nitrogen but also reduce the estimated billions of dollars in annual expenses on nitrogen-based fertilizers worldwide. As a leaf-harvested crop, tobacco leaf traits are often the primary targets for breeders. Lower NUE of tobacco leaves has adverse effects on the chemical matrix, such as the production of higher levels of nitrate and tobacco-specific nitrosamine (TSNA). Considering the negative effects of excess nitrogen on tobacco yield and cured product quality, developing new genotypes with high NUE and productivity under nitrogen deficiency is a priority for breeders (Drake et al., 2015).

NUE comprises two components: nitrogen uptake efficiency (NUpE), which involves the uptake/absorption of available nitrogen, and nitrogen uptake and utilization efficiency (NUtE), which includes nitrogen assimilation and its remobilization (Gojon, 2017; Weih et al., 2018). Plants with enhanced NUE are capable of the coherent uptake, transport, assimilation, and remobilization of nitrogen (Havé et al., 2017; Selvaraj et al., 2017). Therefore, comprehensive insights into nitrogen sensing, uptake, assimilation, and remobilization will provide a crucial basis for improving plant NUE. NUE is a complex trait that is controlled by multiple genes (Fan et al., 2017). Nitrate transporters (NRTs) located on the plasma membranes of root cells participate in nitrate uptake (O'Brien et al., 2016). Following uptake by the root cells, a small amount of nitrate is assimilated into the roots. In contrast, a larger proportion is translocated to the leaves, where it is reduced to ammonium by nitrate reductase (NR) and nitrite reductase (NiR) in the cytoplasm and plastids. Ammonium is assimilated into glutamine (Gln) and glutamate (Glu) by glutamine synthetase (GS) and glutamate synthase (GOGAT) (Tegeder & Masclaux-Daubresse, 2018; Xu et al., 2012).

N reduction and assimilation require both energy and a carbon (C) skeleton, which are generated during photosynthesis and carbon metabolism (Nunes-Nesi et al., 2010; Xu et al., 2012). Nitrogen metabolism also provides N-containing compounds such as enzymes and photosynthetic pigments (Krapp & Traong, 2006). Tremendous progress has been made in unraveling the molecular mechanisms that regulate NUE. Previous studies have shown that NUE can be improved by manipulating candidate genes related to carbon metabolism, nitrogen uptake, transport, and assimilation (Bollam et al., 2021; Li et al., 2018; Zhang et al., 2019). Four families of transporters are involved in nitrate acquisition, translocation, and distribution in plants: the NRT1/peptide transporter (PTR) family (NPF), NRT2, chloride channel family, and the slowly activating anion channel (SLC/SLAH) family (Tegeder & Masclaux-Daubresse, 2018). *NRT1.1* is a dual-affinity NRT and nitrate sensor (Liu et al., 1999). Overexpression of *NtNRT1.1B* markedly enhances tobacco growth and nitrate transport abilities (Wu et al., 2023). *NtNRT2.2/1.2* has also been demonstrated to play an essential role in nitrate distribution and utilization (Liu et al., 2018). Overexpression of another NRT gene, *NtNRT2.11*, greatly improves nitrogen utilization and biomass in tobacco (Wu et al., 2023). In addition to the genes mentioned above, other genes that participate in nitrogen assimilation and reutilization, such as *GS1*, *GS2*, *NR*, *GOGAT*, and *NRT1.7*, are primarily important for regulating nitrogen metabolic pathways and improving NUE in tobacco (Chen et al., 2020; Lu et al., 2016; Wang et al., 2013). In addition, a more detailed study on the effects of thioredoxin (Trx) gene overexpression in chloroplasts showed the importance of Trx in coordinating carbon and nitrogen metabolism in tobacco (Ancín et al., 2021). In another study, overexpression in Nt*NLP7* lines remarkably increased plant growth by coordinately enhancing nitrogen and carbon assimilation, and these attributes correlated with NUE improvement (Yu et al., 2016). Therefore, the expression of these genes may vary among tobacco varieties with different NUEs.

Burley tobacco is deficient in chlorophyll biosynthesis and has a low NUE (Lewis et al., 2012). Consequently, burley tobacco requires much higher quantities of nitrogen fertilization than flue-cured tobacco for comparable yields, which leads to higher nitrate and TSNA accumulation (Lewis et al., 2012). A previous study suggested that carbon and nitrogen traits related to NUE vary between flue-cured and burley tobacco (Li et al., 2017). However, the physiological and molecular factors underlying NUE in burley tobacco are not fully understood. In addition, the mechanisms of lower NUE in burley tobacco have rarely been studied in combination with nitrogen uptake, assimilation, and remobilization. Therefore, this study systematically investigated the different responses of two tobacco genotypes to reduced nitrogen supply in terms of nitrogen uptake, assimilation, and remobilization processes using a combined ^{15}N labeling, physiological, and transcriptomic approach. We identified key metabolic pathways related to nitrogen supply and revealed possible physiological and molecular mechanisms that might drive the differences in NUE between flue-cured and burley tobacco.

2 | MATERIALS AND METHODS

2.1 | Plant materials and growth conditions

The seeds were sterilized twice with 2% (v/v) sodium hypochlorite for 5 min and sown on a sponge in a greenhouse with a 12-h light (28°C)/12-h dark (26°C) photoperiod with approximately 400-μmole m^{-2} s^{-1} photon density and 70% humidity. Flue-cured tobacco K326 from Henan Agricultural University and burley tobacco TN90 from Hubei Tobacco Company were used in the experiment. Seven days after sowing, seedlings (K326 and TN90) were cultured in modified Kimura B solution with NH_4NO_3 as the nitrogen source for

3 weeks (1.25-mM NH_4NO_3 for 1 week followed by 2.5 mM NH_4NO_3 for 2 weeks). Half of the 3-week-old seedlings were transferred to a low-nitrogen solution (.25-mM NH_4NO_3) for 5 days, whereas the other half remained in a high nitrogen condition (2.5-mM NH_4NO_3) for 5 days. The treatments were as follows: leaves of K326 and TN90 were treated with .5 (KL0 and TL0, respectively) or 5-mM NO_3^- (KL5 and TL5, respectively), and roots of K326 and TN90 were treated with .5 (KR0 and TR0, respectively) or 5-mM NO_3^- (KR5 and TR5, respectively). Leaves (up to the bottom and the third leaf from the top) were cut into pieces and frozen immediately in liquid nitrogen for bulk RNA-seq and physiological index measurements.

2.2 | Analysis of the nitrate uptake and transport ability

The 3-week-old seedlings were transferred to the N-free medium for an additional 2 days, after which seedlings were treated with low (.5-mM KNO_3) and high (5-mM KNO_3) nitrate for 2 h. Next, seedlings were treated with .5- or 5-mM ^{15}N-labeled KNO_3 (99% atom ^{15}N-KNO_3, Macklin-Shanghai) in modified Kimura B solution for 3 h and then transferred to unlabeled solution for 3 min and washed in .1-mM $CaSO_4$ for 2 min to remove ^{15}N-NO_3^- from the root surface. Roots and leaves were collected and dried at 65°C. The dried samples were ground, and the ^{15}N content was determined using an isotope ratio mass spectrometer with an elemental analyzer (Thermo Finnigan Delta Plus XP; Flash EA 1112). The ^{15}N content of the whole plant was determined after 3 h of uptake of ^{15}N-KNO_3. The uptake activity was calculated as the amount of ^{15}N taken up per unit weight of the roots per unit of time. Root-to-shoot nitrate transport was determined as the ratio of ^{15}N accumulation (^{15}N mM/g dry weight [DW]) in shoots to that in roots 3 h after ^{15}N-KNO_3 labeling.

2.3 | Analysis of the nitrate remobilization ability

Plants at the five-true-leaf stage were transferred to a 5-mM KNO_3 nutrient solution containing 4.9% excess ^{15}N (99% atom ^{15}N-KNO_3, Macklin-Shanghai) for 1 day. After labeling, tobacco plants were grown with .2-mM unlabeled KNO_3 nutrient solution for 9 days (at the nine-true-leaf stage), and then, individual leaves were collected. The ^{15}N content of the leaves was measured using an isotope ratio mass spectrometer with an elemental analyzer (Thermo Finnigan Delta Plus XP; Flash EA 1112). Growth parameters, enzyme activity related to nitrogen metabolism, nitrate remobilization-related gene expression levels, and leaf amino acid content were measured.

2.4 | Assays of chlorophyll content and enzyme activities

Pigment content was determined by the method described by Zou (Q, 2014). Sucrose synthetase activity, sucrose phosphate synthase (SPS), RuBisCO enzyme activity, NR activity, GS activity, and GOGAT activity were determined using micro-determination kits (Suzhou Comin Biotechnology Co., Ltd, Jiangsu, China).

2.5 | Determination of NO_3–N content and soluble sugars

Samples were placed in an oven at 105°C for approximately 15 min and dried to a constant weight at 65°C. After the DW was recorded, the samples were ground to powder for NO_3–N, and soluble sugar determination. NO_3–N content was determined using the method described by Cataldo et al. (Cataldo et al., 1975). Soluble total sugar and reducing sugar were detected using micro-determination kits (Suzhou Comin Biotechnology Co., Ltd., Jiangsu, China).

2.6 | Measurement of amino acid concentrations

The amino acids were quantified using high-performance liquid chromatography (HPLC). Leaf tissues (.05 g) were extracted with 1 mL of ultrapure water in an Eppendorf (EP) tube. After extraction, the supernatant (200 μL) and an amino acid standard solution (200 μL) were placed into 1.5-mL EP tubes. N-leucine internal standard (20 μL), triethylamine acetonitrile (200 μL) (pH > 7), and phenyl isothiocyanate acetonitrile (100 μL) were added to the samples and incubated at 25°C for 1 h. Next, 400 μL of n-hexane was added to the tube and incubated for 10 min. Finally, the solution was filtered using a .45-μm needle filter and analyzed using an HPLC (Rigol L3000, Beijing, China).

2.7 | Sequencing and functional enrichment analysis of differentially expressed genes (DEGs)

Total RNA was isolated using the TRIzol reagent kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The resulting cDNA library was sequenced by Gene Denovo Biotechnology Co. (Guangzhou, China) using the Illumina Novaseq6000 platform. Quality control of the dataset was performed using the fastp software (version .18.0) (Chen et al., 2018)). Next, the clean sequencing reads were mapped to the K326 tobacco reference genome (https://solgenomics. =organism/Nicotiana_tabacum/genome) using HISAT2. 2.4 (Kim et al., 2015).

The gene expression level of the samples was calculated using fragments per kilobase of transcript per million mapped reads, and RNA differential expression analysis was performed using DESeq2 (Love et al., 2014) software. DEGs were defined using the following criteria: $|\log2(FC)| \geq 1$ and FDR < .05. Venn diagrams, box diagrams, and heat maps were generated using OmicShare, a free online platform for data analysis (https://www.omicshare.com/tools). GO (Ashburner et al., 2000) and KEGG (Kanehisa &

Goto, 2000) pathway enrichment analyses were conducted to analyze the biological functions of the selected modules. Finally, the network file was visualized using the Cytoscape (v3.7.1) (Shannon et al., 2003) software to present biological gene interactions.

2.8 | Statistical analysis

Student's *t*-tests between samples were performed using IBM SPSS Statistics (version 20.0; IBM Corp., Armonk, NY, USA). All data, shown as means ± SD, correspond to the mean of three biological replicates ($n = 3$). GraphPad Prism (v. 8.1, GraphPad Software Inc., San Diego, CA, USA) was used to generate figures.

2.9 | Reverse transcription and real-time PCR

Reverse transcription PCR was performed using reverse transcriptase (Vazyme, Nanjing, Jiangsu, China). qRT-PCR was performed using the SYBR qPCR Master Mix (Vazyme, Nanjing, Jiangsu, China). The primers used are listed in **Supplementary Table S1**. Gene expression was normalized to *EIF5A1*.

3 | RESULTS

3.1 | Overview of RNA-seq data and DEG screening

To investigate the molecular mechanism underlying the difference in nitrogen metabolism between K326 and TN90 cells, high-throughput transcriptomic analysis based on RNA-seq was performed. Global expression profiles under nitrogen-sufficient and nitrogen-starvation conditions were determined in the leaves and roots of K326 and TN90 plants. A total of 12.20, 11.49, 12.41, 13.09, 14.62, 14.45, 15.61, and 12.96 million raw reads were identified in the KL0, KL5, TL0, TL5, KR0, KR5, TR0, and TR5 groups, respectively. After filtering low-quality data, 12.16 (99.74%), 11.46 (99.75%), 12.38 (99.75%), 13.04 (99.61%), 14.58 (99.70%), 14.40 (99.70%), 15.57 (99.71%), and

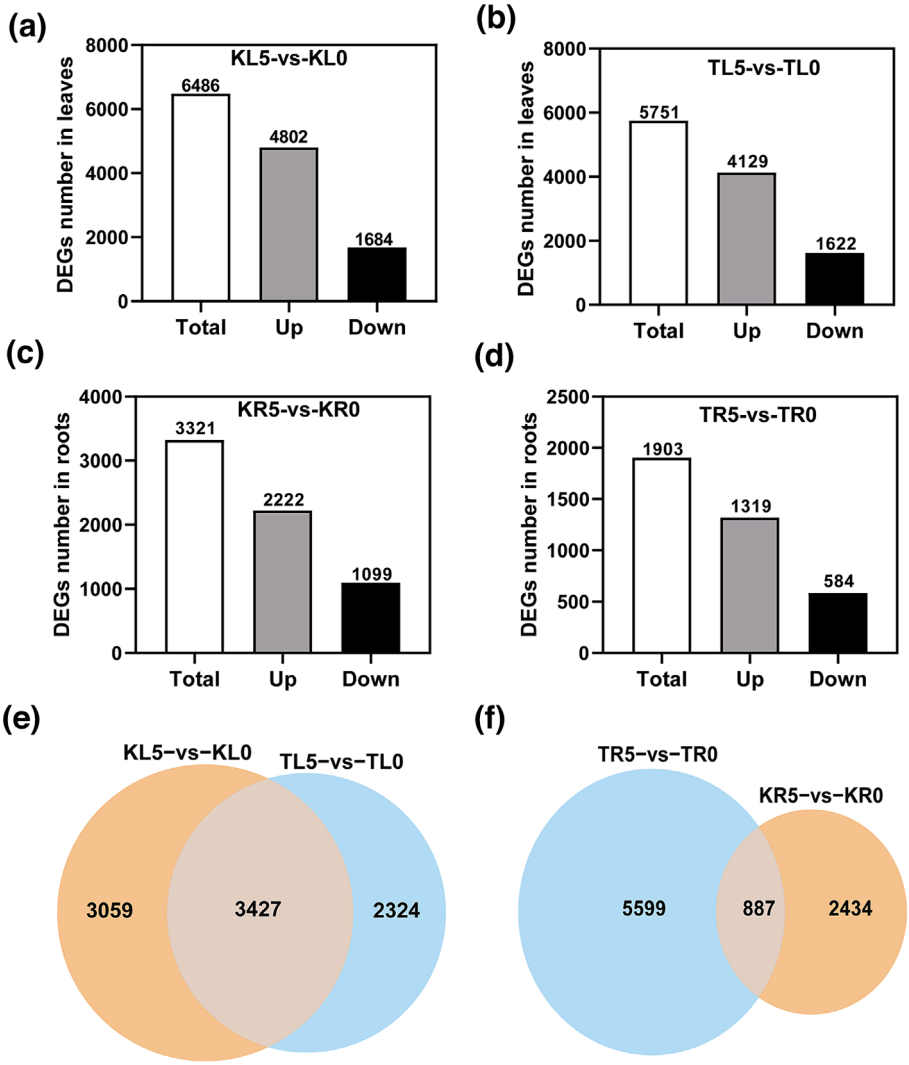

FIGURE 1 Differences in gene expression in leaves and roots of flue-cured tobacco (K326) and burley tobacco (TN90) seedlings in response to low nitrogen supply. (a, b) The number of differentially expressed genes (DEGs) between the normal nitrogen and low nitrogen application in shoots of K326 and TN90. (c, d) The number of DEGs between the normal nitrogen and low nitrogen application in roots of K326 and TN90. (e, f) Venn diagram of DEGs identified in leaves and roots of K326 and TN90 in response to 5-day starvation.

12.92 (99.71%) million clean reads were identified, and the percentage of mapped reads accounted for more than 91% (Table S2). Meanwhile, the quality ≥30 was higher than 92%. In addition, an overview of the gene locations mapped using the RNA-seq reads and cluster analysis of the samples was performed (Figure S1). In summary, the results suggest that the transcriptome data were reliable for subsequent bioinformatics analysis.

DEGs in the leaves and roots were identified by comparing the two nitrogen treatments (KL5/KL0 and TL5/TL0). The results showed that there were 6486 (leaves) and 3321 (roots) DEGs in K326 and 5751 (leaves) and 1903 (roots) DEGs in TN90 (Figure 1a–d). The Venn diagram showed that 3427 common DEGs in the leaves and 887 in the roots were screened in the two cultivars in response to nitrogen deficiency stress (Figure 1e,f). Common DEGs are the genes most likely associated with the low-nitrogen stress response.

3.2 | Functional analysis of the common DEGs between tobacco genotypes in response to nitrogen supply

GO and KEGG enrichment analysis of the common DEGs was conducted to further reveal the transcriptional mechanism of the tested tobacco genotypes in response to nitrogen deficiency. DEGs were annotated based on three major categories, that is, "cellular component," "molecular function," and "biological process." The results showed that the top GO terms in the "biological process" and "cellular component" categories were enriched in "plastid part" and "response to acid chemical" in leaves of K326 and TN90 (Table S3; Figure 2a). In roots of K326 and TN90, the top GO terms in the "biological process," "molecular function," and "cellular component" categories were enriched in "nitrate metabolic process," "inorganic anion transmembrane activity," and "intrinsic component of membrane"

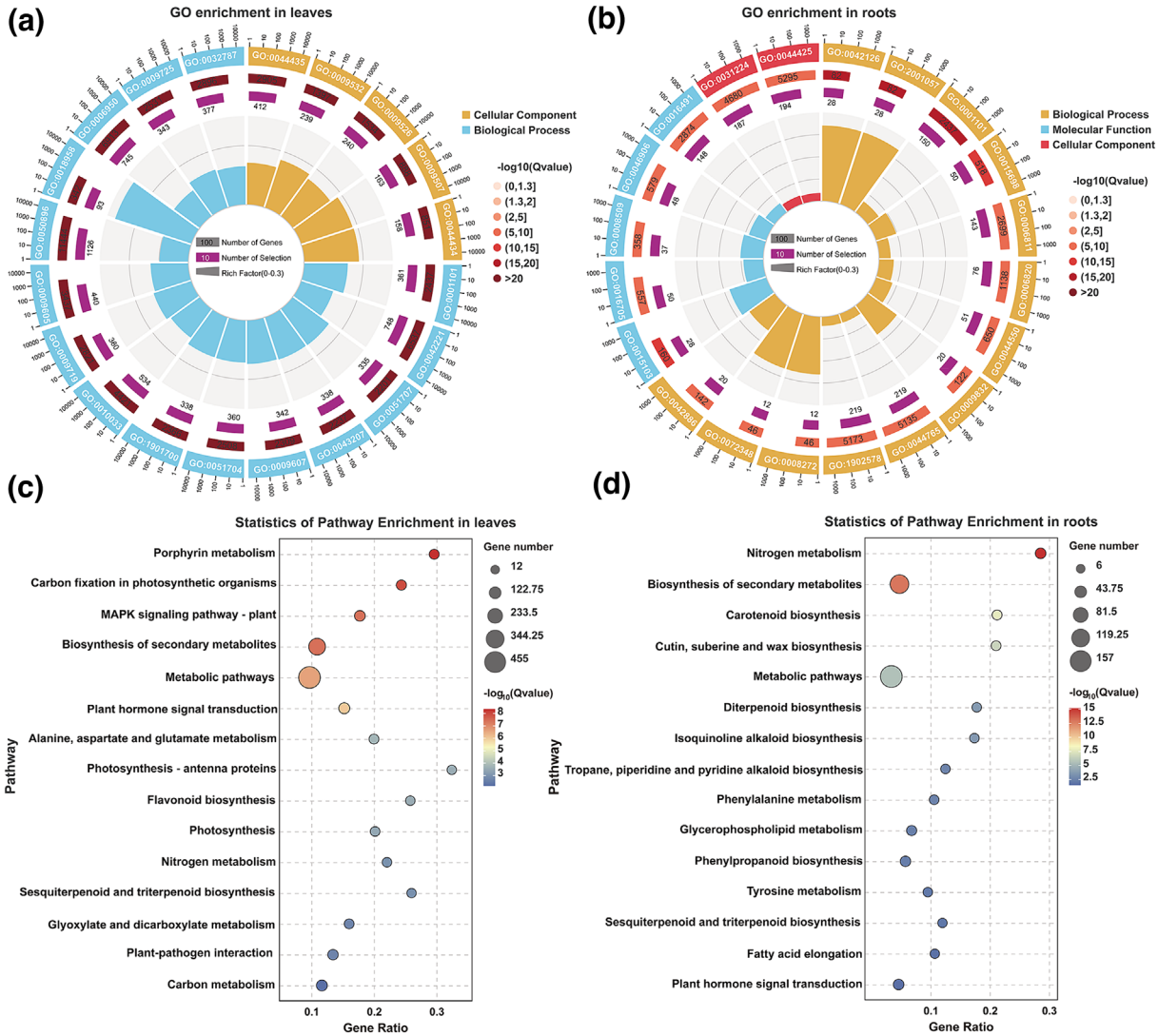

FIGURE 2 GO and KEGG pathway enrichment of the common DEGs in leaves and roots of two tobacco genotypes with normal and low nitrogen supply. (A-B) GO term's enrichment statistical circle chart (top 20) of the common DEGs in leaves and roots. (c, d) Top 15 KEGG pathway analysis of the common DEGs in leaves and roots.

(Table S3; Figure 2b). KEGG enrichment analysis revealed that the common DEGs in leaves mainly functioned in "porphyrin metabolism," "carbon fixation in photosynthetic organisms," "plant hormone signal transduction," "alanine, aspartate, and glutamate metabolism," "photosynthesis," and "nitrogen metabolism" (Figure 2c). The common DEGs in the roots mainly functioned in "nitrogen metabolism" and "carotenoid biosynthesis" (Figure 2d).

3.3 | Transporters that were differentially regulated by nitrogen supply between K326 and TN90

Nitrate, a major source of nitrogen in the environment, is absorbed and transported by NRTs for assimilation and storage. All DEGs common between K326 and TN90 encoding transporters were identified. In total, 22 transporter genes in the leaves and 35 transporter genes in the roots were identified, including the *NRT2* family, NRT1/NPF, and *transcription factor NIN-like protein* (*NLP*) (Table S4). We found that the expression levels of most NRT genes did not differ between K326 and TN90. However, three *NPF2* genes (*gene_31129*, *gene_44740*, and *gene_68854*) and one *NPF3* gene (*gene_3817*, *NPF3.1*) in the leaves were significantly up-regulated by nitrogen starvation, and two *NPF6* genes (*gene_45098* and *gene_82596*) were down-regulated by nitrogen starvation, all of which showed greater expression in K326 than in TN90 (Table S4; Figure 3a). In addition, three transporters that were up-regulated by nitrogen deficiency stress and six transporters that were down-regulated by nitrogen deficiency stress in the roots exhibited higher expression in K326 than in TN90 (Table S4; Figure 3b). These screened genes may play important roles in conferring the differential NUE between K326 and TN90.

3.4 | KEGG enrichment of common up and down-regulated DEGs in leaves and roots

The 3427 and 887 DEGs common between the leaves and roots of the tobacco genotypes, respectively, and the common up-regulated and down-regulated DEGs were further analyzed (Figure S2). KEGG pathway enrichment analysis showed that the common down-regulated DEGs in leaves were mainly enriched in pathways related to

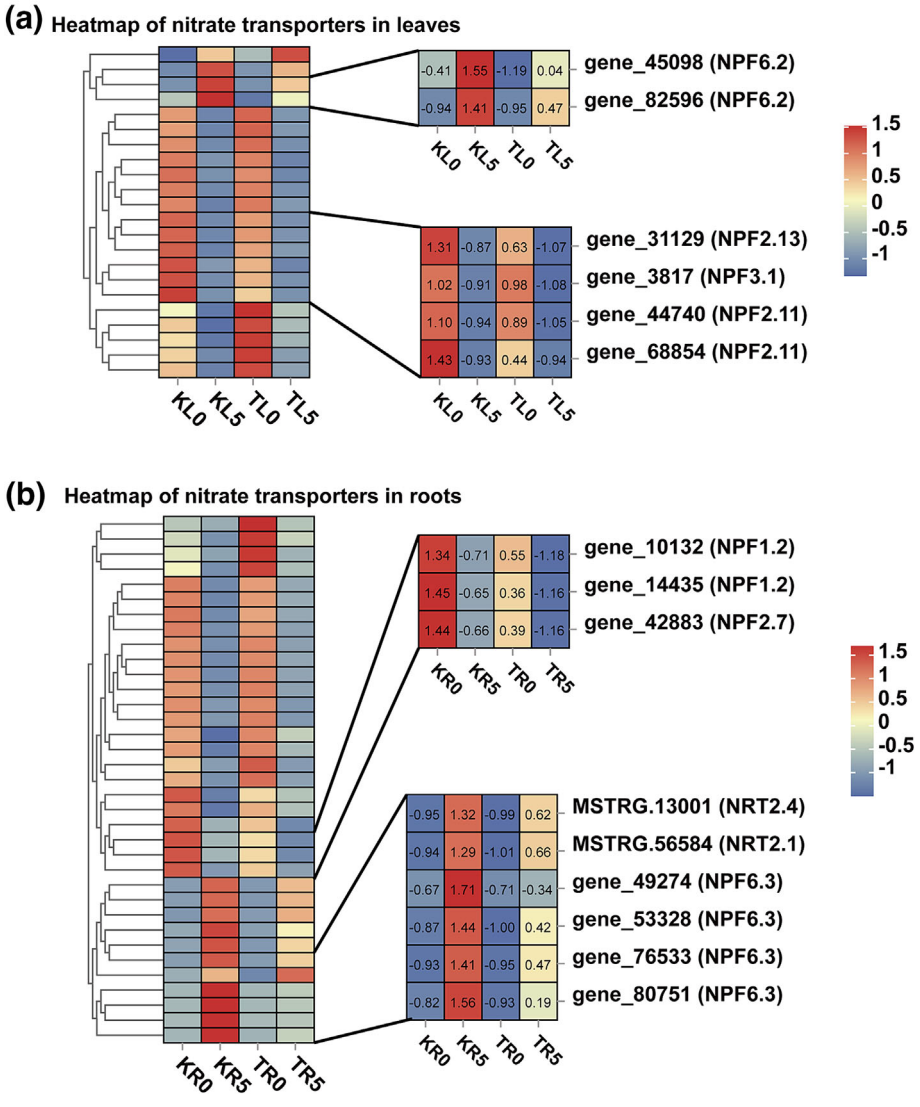

FIGURE 3 Transporter genes were identified using RNA-seq in leaves and roots of K326 and TN90 under normal and low nitrogen treatments. (a) Heat maps of nitrate transporter genes in leaves of K326 and TN90 under the normal and low nitrogen application conditions. (b) Heat maps of nitrate transporter genes in roots of K326 and TN90 under the normal and low nitrogen application conditions.

carbon and nitrogen metabolism, such as "carbon fixation in photosynthetic organisms," "porphyrin metabolism," "carbon metabolism," "photosynthesis," "photosynthesis-antenna proteins," and "nitrogen metabolism," whereas the common up-regulated DEGs were mainly enriched in "plant hormone signal transduction," and "MAPK signaling pathway" (Figure 4a,b). In roots, the most activated enriched pathway was "nitrogen metabolism," whereas the most inhibited pathway was "carotenoid biosynthesis" (Figure 4c,d).

It is well documented that carbon and nitrogen metabolism are closely coupled in plants, and previous studies have shown that the expression of many genes correlated with carbon and nitrogen metabolism was changed under different nitrogen conditions (Li et al., 2017). Therefore, we focused our subsequent analyses on common down-regulated DEGs related to carbon and nitrogen metabolism. Notably, we found that most of the down-regulated genes showed higher levels of expression in K326 than in TN90 under normal or low nitrogen supply, which are possible factors related to the inhibition of growth of tobacco seedlings under nitrogen deficiency stress and a lower NUE in TN90 than in K326 (Figure S3). However, all DEGs associated with photosynthesis-antenna proteins showed lower expression levels in K326 than in TN90, which may be a compensatory reaction to nitrogen starvation in TN90 leaves (Figure S3). In roots, most of the DEGs mapped in nitrogen metabolism were down-regulated with nitrogen starvation and had higher expression levels in K326 than in TN90 under normal or low nitrogen supply (Figure S3). These results suggest that the efficient maintenance of carbon and nitrogen metabolism is conducive to the adaptability of tobacco plants to low-nitrogen stress.

3.5 | Identification of key DEGs associated with carbon and nitrogen metabolism using protein–protein interaction (PPI) network construction

We identified 17 DEGs in carbon fixation in photosynthetic organisms, 23 DEGs in porphyrin metabolism, 31 DEGs in carbon

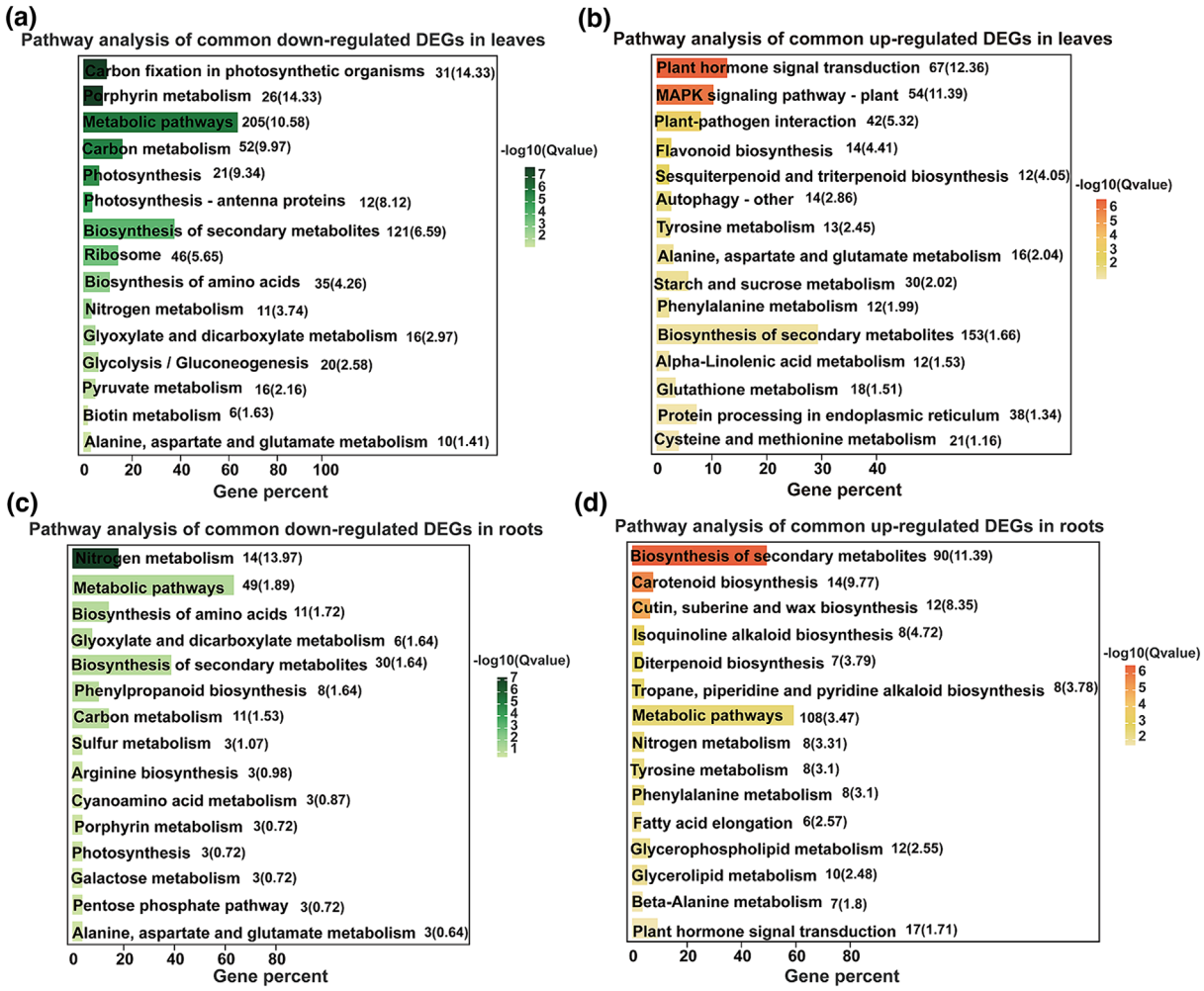

FIGURE 4 The statistics of KEGG enrichment of common up- and down-regulated DEGs in leaves and roots of flue-cured tobacco (K326) and burley tobacco (TN90) seedlings in response to low nitrogen supply. KEGG pathway analysis of common up and down-regulated DEGs in leaves (a, b) and roots (c, d) of flue-cured tobacco (K326) and burley tobacco (TN90) seedlings in response to low nitrogen supply. The X-axis indicates the gene percentage. The column color and length indicate the Q value and gene number.

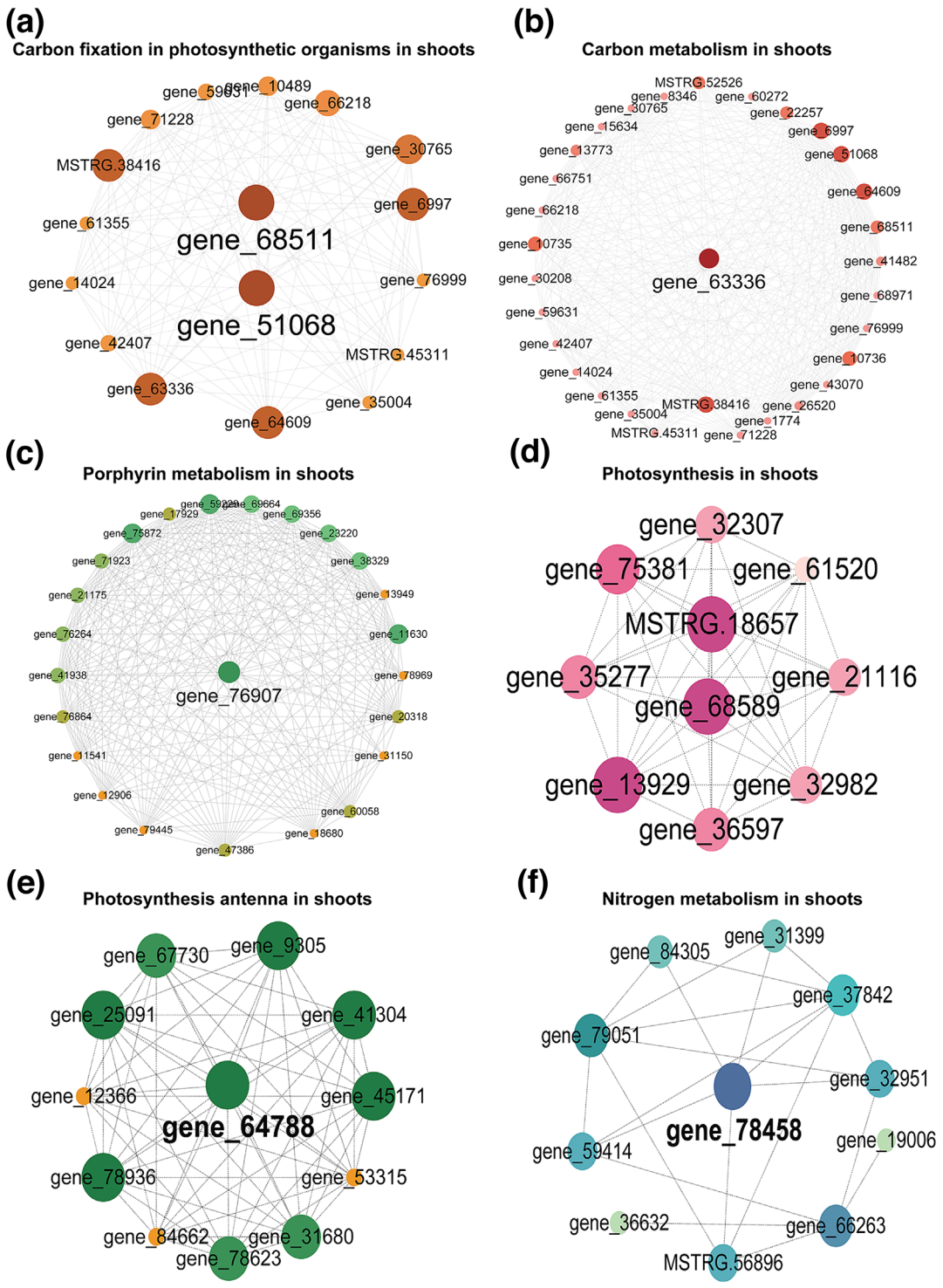

FIGURE 5 PPI network of genes related to key metabolism pathways in leaves and roots. PPI network of genes related to carbon fixation photosynthetic organisms (a) and carbon metabolism (b), porphyrin metabolism (c) and photosynthesis (d), and photosynthesis antenna (e) and nitrogen metabolism (f) in leaves. The circles' size and color represent the power of the interrelations among the nodes by degree value.

metabolism, 10 DEGs in photosynthesis, 12 DEGs in photosynthesis-antenna proteins, and 11 DEGs in nitrogen metabolism in leaves from the results of the KEGG analysis of the common down-regulated DEGs (Figure S3). We speculated that these screened DEGs are important in improving resistance to low nitrogen stress and maintaining NUE. A PPI network was constructed to further identify the key genes in key metabolic pathways. The network diagram indicated that *gene_51068* (*TPIP1*) and *gene_68511* (*GAPB*) in carbon fixation in photosynthetic organisms; *gene_76907* (*HEMB*) in porphyrin metabolism; *gene_63336* (*PGK3*) in carbon metabolism; *gene_13929*

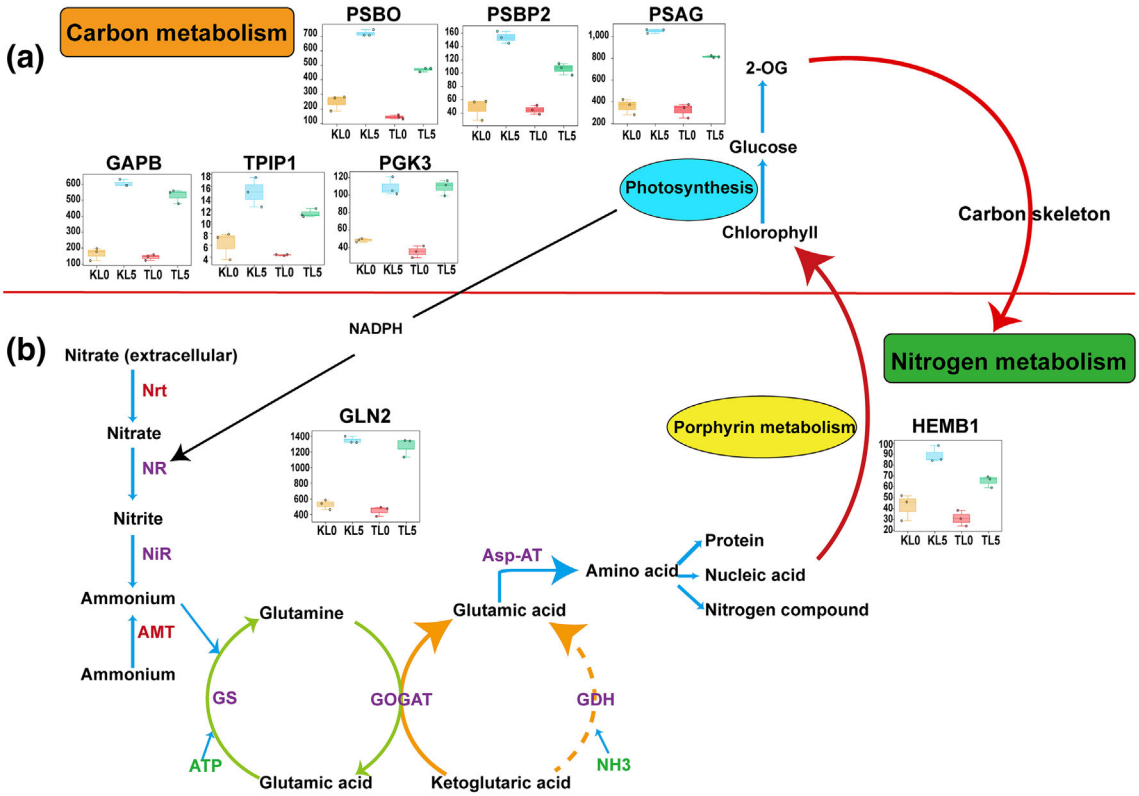

FIGURE 6 The relationship between carbon and nitrogen metabolism and changes in key gene expression in the leaves of K326 and burley tobacco TN90. (a) Carbon metabolism process. (b) Nitrogen metabolism process. The box diagram shows the key gene expression level in carbon and nitrogen metabolism. The red arrows represents the relationship between carbon and nitrogen metabolism. For each treatment, 15 tobacco plants were selected. Five tobacco seedlings from each treatment were blended as biological replicates, and three replicates of each treatment were included in the study.

(*PSBO*), *gene_68589* (*PSBP2*), and *MSTRG.18657* (*PSAG*) in photosynthesis; and *gene_78458* (*GLN2*) in nitrogen metabolism in leaves had higher degree values than the other genes (Table S5; Figure 5a–f). In addition, we analyzed the expression patterns of key genes related to carbon and nitrogen metabolism as described above. Notably, we found that these key genes were down-regulated under low-nitrogen conditions and exhibited higher expression levels in K326 leaves than in TN90 leaves (Table S5; Figure 6). Carbon metabolism is inhibited by nitrogen deficiency, producing fewer carbon skeletons and NADPH for nitrogen metabolism (Figure 6). There was a lower capacity for photosynthesis and carbon fixation in TN90 leaves than in K326 leaves, which hampered nitrogen metabolism and resulted in a lower NUE than in K326.

3.6 | Validation of DEGs by quantitative real-time PCR (qRT-PCR)

Eight DEGs in the leaves and four DEGs in the roots were selected for qRT-PCR analysis to further verify the reliability of the transcriptome data (Figure S4). The expression patterns of the 12 genes detected using qRT-PCR were consistent with transcriptome expression trends. These results indicate that the transcriptome data were valid and reliable.

3.7 | Differences in the carbon/nitrogen (C/N) physiological traits between K326 and TN90

The growth of the tobacco plants was significantly inhibited when they were exposed to nitrogen deficiency (Figure 7a). Leaf and root biomass was lower in plants starved of nitrogen than those grown in N-sufficient conditions; however, the biomass was greater in K326 than in TN90, regardless of the nitrogen supply (Figure 7b,c). As mentioned previously, "carbon fixation," "photosynthesis," and "porphyrin metabolism" were enriched in DEGs in leaves in K326 and TN90 (Figure 2c). The results showed that chlorophyll content was reduced in plants starved of nitrogen, whereas K326 maintained a higher content than TN90 under both nitrogen supplies (Figure 7d,e). Similarly, the enzyme activities of Rubisco, SS, and SPS were decreased in nitrogen-deficient conditions but were higher in K326 than in TN90 under both nitrogen levels (Figure 7f–h). In contrast, the concentrations of total sugar and reducing sugar in the leaves were greater in N-starved plants than in nitrogen-sufficient plants for both genotypes

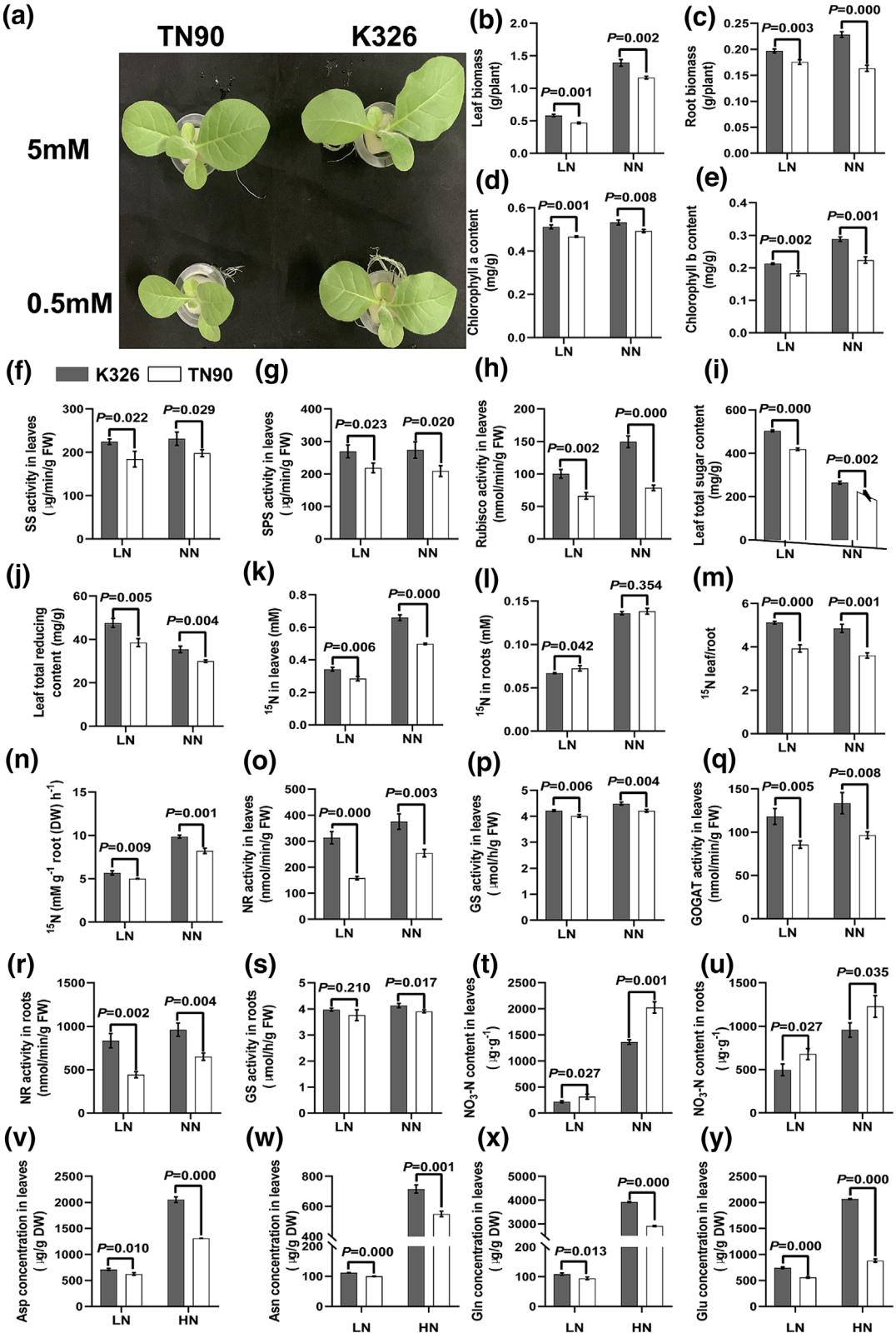

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FIGURE 7 Analysis of morphological parameters correlated with carbon and nitrogen metabolism in leaves and roots of flue-cured tobacco (K326) and burley tobacco (TN90) seedlings grown under normal (HN) and low (LN) nitrogen levels. (A) the phenotype of K326 and TN90 tobacco seedlings under different levels of nitrogen application. (b, c) Leaf and root biomass. (d) Chlorophyll a. (e) Chlorophyll b. (f–h) SS, SPS, Rubisco activity in leaves. (i, j) Total sugar and reducing sugar content. (k, l) ^{15}N content in leaves and roots. (m) Nitrate uptake activity of K326 and TN90. (n) Results of nitrate root-to-shoot transport assay of K326 and TN90. (o–q) NR, GS, GOGAT activity in leaves. (r) NR activity in roots. (s) GS activity in roots. (t) NO_3–N content in leaves. (u) NO_3–N content in roots. Asp concentration in leaves (v), Asn concentration in leaves (w), Gln concentration in leaves (x), and Glu concentration activity in leaves (y). For each treatment, 15 tobacco plants were selected. Five tobacco seedlings from each treatment were blended as biological replicates, and three replicates of each treatment were included in the study. Values are means ± SD, and significant differences were calculated using a two-tailed Student's *t*-test.

(Figure 7i,j). Overall, the results revealed that K326 had a significantly higher tolerance to N-limitation than TN90.

To understand the mechanisms impacting NUE in K326 and TN90, a ^{15}N-nitrate-labeled hydroponics experiment was performed in which .5- and 5-mM ^{15}N-nitrate were provided. The results showed that nitrogen deficiency led to a decrease in leaf ^{15}N accumulation, nitrate uptake activity, and nitrate root-to-shoot transport; these were higher in K326 than in TN90 under both nitrogen supply levels (Figure 7k–n). However, the ^{15}N content in the roots accumulated to a much greater extent in TN90 than in K326 under both nitrogen levels (Figure 7l). Generally, K326 exhibited better nitrate uptake and transport performance than TN90. Additionally, nitrogen deficiency led to reduced NR, GS, and GOGAT activity in the leaves of both K326 and TN90 plants but was greater in K326 plants than in TN90 plants under various nitrogen conditions (Figure 7o–q). Similar changes were observed in the enzyme activities of NR and GS in roots but were higher in K326 than in TN90 under both N-sufficient and N-deficient conditions (Figure 7r,s). Furthermore, we detected NO_3–N content in the leaves and roots of K326 and TN90. As shown in Figure 7t,u, the NO_3–N content in the leaves and roots of K326 was lower than that of TN90 under both normal and low nitrogen conditions, indicating that K326 had a higher capability for nitrate reduction and assimilation than TN90. The KEGG analysis showed that "nitrogen metabolism" and "biosynthesis of amino acids" pathways were inhibited in leaves responding to nitrogen deficiency between the two tobacco genotypes (Figure 4a). The concentrations of free amino acids, including Glu, Gln, aspartate (Asp), and asparagine (Asn) in the leaves of the two genotypes of tobacco seedlings receiving different nitrogen levels, were analyzed to study the metabolites involved in this pathway in detail. The results showed that Glu, Gln, Asp, and Asn were more abundant in K326 than in TN90 plants when plants were exposed to nitrogen deficiency stress (Figure 7v–y).

3.8 | Leaf biomass and nitrate remobilization characteristics at different parts of tobacco leaves under nitrogen starvation conditions

We investigated the growth behavior of individual leaves of K326 and TN90 tobacco plants under nitrogen starvation conditions to investigate the effects of long-term nitrogen starvation on the growth and nitrate remobilization from old to young leaves. As shown in Figure 8a, there was a remarkable difference in leaf area and size between the K326 and TN90 plants. The DWs of large and young leaves in K326 increased by 25% and 81%, respectively, relative to those of TN90 (Figure 8b). Furthermore, higher ^{15}N accumulation and the proportion of ^{15}N were allocated to the large and young leaves of K326 plants relative to TN90 (Figure 8c,d). The contents of Chl a and Chl b in the large and young leaves of K326 were significantly higher than those of TN90 (Figure 8e,f). These results imply that K326 exhibited a higher NUE than TN90 and could more effectively allocate limited nitrogen resources to young leaves to enable plants to grow much better.

We analyzed nitrogen metabolism enzyme activities and key gene expression levels in different parts of the leaves to determine whether there was a difference in nitrogen assimilation and reutilization between the two tobacco genotypes. Notably, K326 leaves retained higher NR, NiR, GS, and GOGAT enzyme activities than TN90 (Figure 8g–j). In addition, higher expression levels of *GS1–2*, *GOGAT1*, and *NRT1.7*, which are critical for nitrogen recycling and remobilization (Chen et al., 2020; Li et al., 2017), were observed (Figure 8k–m). Other changes in metabolism during tobacco starvation included higher levels of Gln, Asp, and Asn in young leaves of K326 than in the leaves of TN90 (Figure 8n–q). Asn and Asp, on the other hand, were significantly lower in old leaves of K326 than in TN90 (Figure 8n,p). These results suggest that K326 leaves display better nitrogen assimilation efficiency and source-to-sink nitrogen remobilization from old to young leaves under nitrogen starvation.

4 | DISCUSSION

4.1 | Physiological characteristics that contribute to high NUE in tobacco

Improving the NUE is urgently required to sustain agriculture and protect the environment. In recent years, many studies have been conducted to improve the NUE of crops (Drake et al., 2015; Meng et al., 2021; Wu et al., 2023). Tobacco is used as a model plant and an economic crop. It consumes a relatively large nitrogen number of nitrogen fertilizer to obtain optimal growth and yield, but more than half of the applied nitrogen is lost to the environment because of deficient nitrogen assimilation and utilization (Fan et al., 2018). Therefore, identifying the physiological and molecular mechanisms contributing to NUE is key to solving this problem. In the current study, both short- and long-term hydroponic experiments were conducted to

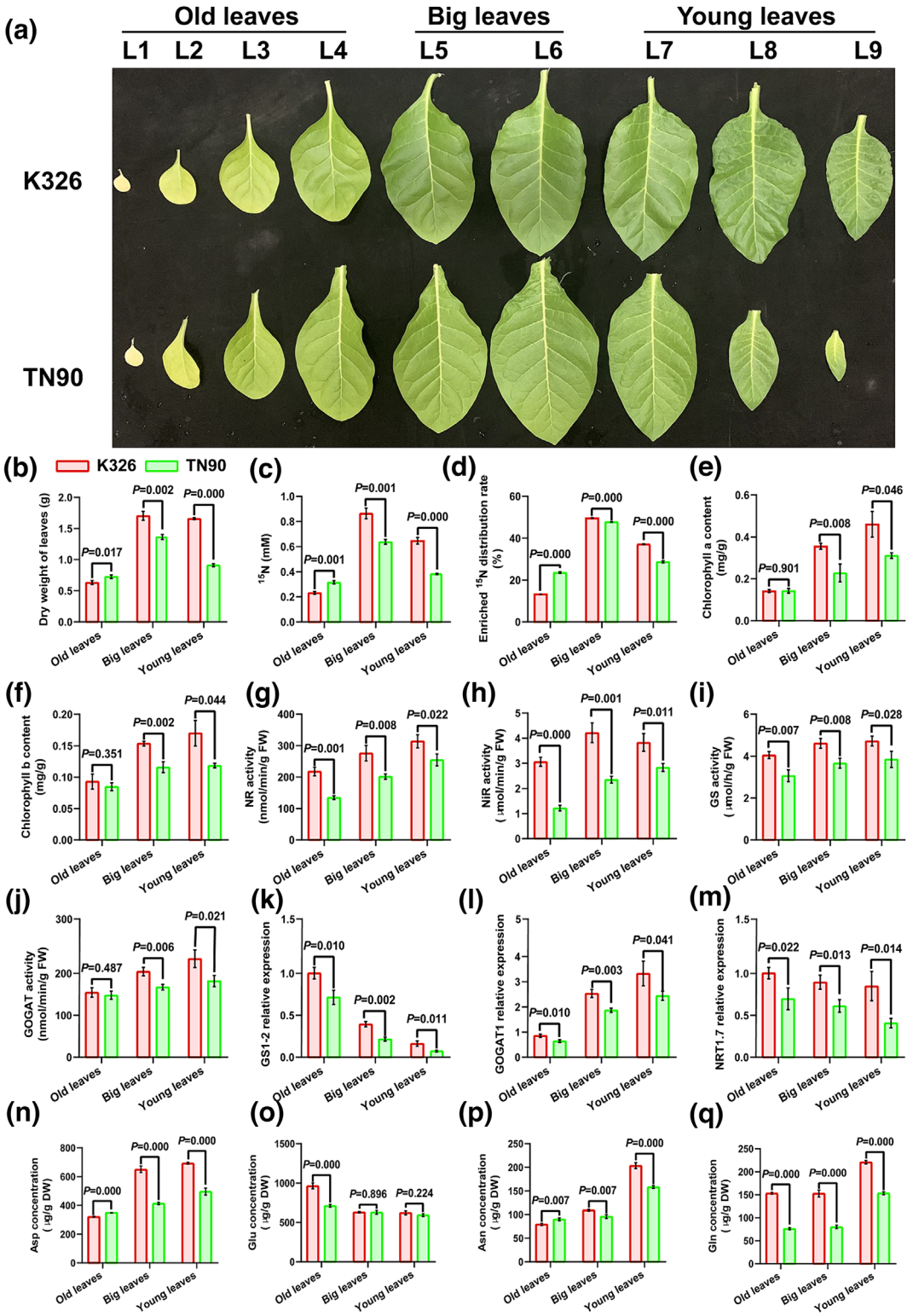

FIGURE 8 N remobilization characteristics at different parts of flue-cured tobacco (K326) and burley tobacco (TN90) leaves under nitrogen starvation conditions. (a) Representative nine-true-leaf stage tobacco leaves grown hydroponically under conditions of nitrogen fluctuation. L1–L9 represents true leaf numbers, with L1 being the oldest true leaf and L9 the youngest leaf. (b) Dry weight of different leaves. (c, d) Distribution of ^{15}N and ^{15}N distribution rate in different leaves. (e, f) Chlorophyll a and b content in different leaves. (g–q) NR activity (g), NiR activity (h), GS activity (i), GOGAT activity (j), GS1–2 relative expression (k), GOGAT1 relative expression (l), NRT1.7 relative expression (m), asp concentration (n), Glu concentration (o), Asn concentration (p), and Gln concentration (q) in different leaves. For each treatment, 15 tobacco plants were selected. Five tobacco seedlings from each treatment were blended as biological replicates, and three replicates of each treatment were included in the study. Values are means ± SD, and the significant differences were calculated using a two-tailed Student's *t*-test.

clarify the mechanisms underlying NUE and the factors contributing to lower NUE in burley tobacco.

Previous studies have indicated that improvements in NUE are required to simultaneously improve NupE and NutE (Kiba & Krapp, 2016). In the short-term root uptake assays using ^{15}N-NO_3^- as a tracer, the N-inefficient line TN90 had lower ^{15}N accumulation, nitrate uptake activity, and nitrate root-to-shoot transport than the N-efficient line K326 under both normal and low nitrogen conditions (Figure 1k–n). K326 had greater leaf and root biomass than TN90 when treated with different nitrogen concentrations for 5 days (Figure 1b,c). Our results showed that K326, with a high NUE, had a higher nitrogen uptake efficiency than TN90, which is in line with previous results for tobacco (Teng et al., 2015), wheat (Shi et al., 2022), and *Brassica napus* (Li et al., 2020). Our hydroponic experiment showed that K326 had a much higher root biomass than TN90 under low nitrogen supply (Figure 1c), which might lead to low nitrogen uptake ability and NUE.

In addition to differences in nitrogen uptake efficiency, nitrogen assimilation, and remobilization of plant nitrogen from old leaves to newly growing tissues are two key components of NUE (Tegeder & Masclaux-Daubresse, 2018). NR and GS are the two most important enzymes associated with nitrogen assimilation (Tegeder & Masclaux-Daubresse, 2018). Ammonium taken up by AMTs or derived from nitrate is used to produce a variety of amino acids, such as Glu, Gln, Asn, and Asp, via the GS/GOGAT cycle (Xu et al., 2012). The nitrogen-efficient line K326 had greater NR, GS, and GOGAT activities than the nitrogen-inefficient line TN90 under both normal nitrogen supply and nitrogen starvation (Figure 1o–q), indicating that K326 was able to retain a greater nitrogen assimilation capacity under these conditions. This was proven by the higher concentrations of Asp, Asn, Glu, and Gln and lower NO_3–N in K326 leaves than in TN90 leaves (Figure 1t–y). Therefore, greater nitrogen assimilation might also contribute to enhanced NUE in tobacco, which is consistent with previous studies on rice (Lee, 2021) and *B. napus* L. (Li et al., 2018).

In plants, nitrogen can be remobilized in inorganic forms such as nitrates. Nitrate is transported via the xylem driven by transpiration. Expanded leaves with stronger transpiration yield more nitrate from the xylem than smaller young leaves do. A recent study revealed that nitrate remobilization from old to young leaves might be an important contributor to NUE (Chen et al., 2020). In this study, we found that less ^{15}N accumulated in old leaves and more ^{15}N was distributed to younger leaves in K326 than in TN90 (Figure 1c,d). In addition, K326 had a greater biomass of young leaves than TN90 (Figure 1a,b), indicating that K326 exhibited more efficient nitrate remobilization than TN90. It is common knowledge The GS and GAGOT enzymes play a central role in nitrogen remobilization (Cánovas et al., 2018; Lee et al., 2020). Furthermore, the GS/GOGAT cycle is responsible for the production of Asn and Gln, the preferential exported forms essential for nitrogen recycling (Lee et al., 2020). The results of this study suggested that K326 had greater GS and GOGAT enzymatic activity than TN90, suggesting that K326 displayed a greater capacity for amino acid biosynthesis (Figure 1i–j). This was corroborated by the greater concentrations of Gln and Asn accumulating in the young leaves of K326 compared with those of TN90 (Figure 1p,q). These data suggest that K326 has a greater ability for nitrogen utilization and remobilization under nitrogen starvation conditions, which may contribute to enhanced NUE in K326. Previous studies have reported that amino acids transferred by transmembrane amino acid transporter proteins are one of the predominant forms of nitrogen remobilization, and their efficient transportation is essential for the growth and development of young organs (Tegeder & Masclaux-Daubresse, 2018). The amino acid transporters and the ability to transfer amino acids in K326 and TN90 require further experimental evidence.

It is common knowledge that photosynthesis and carbon metabolism function to supply nitrogen assimilation with both energy and carbon skeletons (Nunes-Nesi et al., 2010; Xu et al., 2012). The chlorophyll content and RuBisCO, SS, and SUS enzyme activities in the leaves decreased under nitrogen starvation, whereas they were greater in K326 than in TN90 (Figure 1d–h). Similar results were observed for total sugar and reduced sugar content (Figure 1i–j). The results of the present study indicate that the N-efficient genotype has a greater ability to assimilate carbon than the nitrogen-inefficient genotype. Consistent with previous research (Wang et al., 2015; Zhang et al., 2018), these results demonstrate that the C/N balance, which is critical for achieving optimal plant growth, varied differently between the two tobacco genotypes with diverse NUE. Based on the functional analysis of photosynthetic organs, nitrogen can be divided into two types: light capture and bioenergetics. Rubisco, a bioenergetic compound, stores a large amount of nitrogen in these photosynthetic proteins, and the weakening of these enzyme activities reduces the overall amount of carbon fixed by the plant (Nunes-Nesi et al., 2010). Thus, our study indicates that stronger photosynthetic capacity in the low–nitrogen–tolerant cultivar indicates more carbon fixation to accelerate nitrogen uptake, assimilation, and remobilization, which is a means for improving NUE (Masclaux-Daubresse et al., 2010).

In general, the physiological results of the present study revealed that the efficient genotype was more efficient in carbon fixation, nitrogen uptake, nitrogen utilization, and nitrogen remobilization than the inefficient genotype. Plants respond to physiological nitrogen supply through changes in the transcription of related genes, resulting in changes in the activities of key enzymes and metabolite concentrations that cause feedback regulation of nitrogen uptake, assimilation, and reutilization (Xu et al., 2012). Therefore, further analyses of the genes related to the corresponding processes and pathways were conducted.

4.2 | Transcriptomic characteristics that contribute to high NUE in tobacco

NUE comprises two key components: NupE and NutE (Gojon, 2017; Weih et al., 2018). The physiological studies reported here indicated that the N-efficient genotype had greater nitrogen uptake and transport abilities than the N-inefficient genotype (Figure 1k–n). Few studies have focused on nitrogen uptake and translocation in different

plants and have identified transporters that are considered important for enhancing NUE in future studies (Li et al., 2020; Tegeder & Masclaux-Daubresse, 2018). GO analysis of the root transcriptomes of the two genotypes indicated that GO terms related to nitrate metabolic processes and inorganic anion transmembrane activity were enriched in the DEGs that responded to nitrogen deficiency (Figure 2b). Previous studies in *Arabidopsis thaliana* reported that the expression of some *NRT2* transporters and *NPF6.3* genes was induced under N-deficient conditions (Ruffel et al., 2021; Sakuraba et al., 2021). In this experiment, two high-affinity NRTs (*NRT2.1* and *NRT2.4*) and four dual-affinity NRTs (*NPF6.3*) were repressed by nitrogen deficiency stress (Figure 3b), which may be due to differences in experimental materials. Moreover, all of them exhibited higher expression in K326 cells than in TN90 cells (Figure 3b), suggesting their roles in nitrogen uptake and translocation and in enhancing NUE in tobacco.

Some physiological traits that affect NUE, including enzyme activities related to carbon fixation, nitrogen metabolism, and nitrogen remobilization, are modulated by the expression of many genes (Ancín et al., 2021; Chen et al., 2020). In this study, we found that most of the common down-regulated DEGs in leaves were associated with carbon fixation in photosynthetic organisms, porphyrin metabolism, carbon metabolism, photosynthesis, and nitrogen metabolism under nitrogen starvation and showed higher levels of expression in K326 than in TN90 under normal or low nitrogen supply (Figure 4a), which inhibited the photosynthesis rate and nitrogen utilization. This was in accordance with the physiological differences between the two genotypes. Similar results have been reported for other species, such as *B. napus* L. (Li et al., 2020), and rice (Lee, 2021). In recent years, many researchers have focused on screening functional genes associated with nitrogen utilization, leading to the discovery of numerous nitrogen-associated genes in many plant species (Li et al., 2020). For instance, it was demonstrated that Arabidopsis *NPF2.11* and *NPF2.13* are involved in nitrate uptake and transport processes (Liu et al., 2023; Wu et al., 2023). Rice *NRT1.7* and *NPF7.9* genes are responsible for remobilizing stored nitrate and delivering it into source leaves (Chen et al., 2020; Guan et al., 2022), and an important role of *NiR1*, *GOGAT*, and *GLN* genes in nitrogen assimilation and utilization has been observed (Bi et al., 2017; Gao et al., 2019; Konishi et al., 2018). Previous findings have suggested that nitrogen deficiency can strongly influence the expression of N-responsive genes (Li et al., 2020; Shi et al., 2022; Sun et al., 2021). In the present study, a small number of key genes were identified using PPI network analysis (Figure 5). Similarly, our data indicated that *NIR1* (*gene_84305* and *gene_31399*), *GLN1–1* (*gene_37842*), and *GLN2* (*gene_78458* and *gene_79051*) were repressed under nitrogen starvation conditions and that K326 maintained a relatively higher expression than TN90 (Figure S3). In addition, higher expression levels of *GS1–2*, *GOGAT1*, and *NRT1.7* were observed in the young leaves of K326 than in those of TN90 (Figure 8k–m). Given the gene functions, our results suggest that nitrogen utilization-related genes contribute to low nitrogen stress responses and different NUE between tobacco genotypes.

Plant nitrogen is stored in photosynthetic proteins and chlorophyll; therefore, there is a strong relationship between carbon and nitrogen metabolism (Duan et al., 2018; Naliwajski & Skłodowska, 2018). Our transcriptome data indicated that many down-regulated DEGs were associated with photosynthesis, carbon fixation, and carbon metabolism (Figure 4a). Furthermore, several hub genes associated with carbon metabolism and photosynthesis were identified through PPI network analysis, such as *gene_51068* (*TPIP1*) and *gene_68511* (*GAPB*) in carbon fixation, *gene_76907* (*HEMB*) in porphyrin metabolism, *gene_63336* (*PGK3*) in carbon metabolism, *gene_13929* (*PSBO*), *gene_68589* (*PSBP2*), and *MSTRG.18657* (*PSAG*) in photosynthesis (Figure 5a–f). More importantly, we found that these nitrogen stress-related genes were more highly expressed under nitrogen deficiency in the nitrogen-inefficient NUE cultivar K326 than in the nitrogen-deficient NUE cultivar (Figure S3). Our research indicates that K326 can maintain a higher level of nitrogen to support chlorophyll, protein synthesis, and photosynthesis to resist low-nitrogen stress, thus leading to a higher NUE in K326 than in TN90 (Mauceri et al., 2021; Sun et al., 2021). These results suggest that efficient maintenance of carbon and nitrogen metabolism contributes to resistance to low-nitrogen stress and NUE enhancement in tobacco.

5 | CONCLUSION

In conclusion, our study showed that chlorophyll content, nitrogen uptake and transport efficiency, and enzyme activities related to carbon fixation and nitrogen were significantly reduced after exposure to nitrogen deficiency but were higher in K326 than in TN90. In addition, nitrogen deficiency caused more transcripts to be down-regulated, particularly those associated with the porphyrin, photosynthesis, and carbon and nitrogen metabolism pathways. Plant NUE is a complex quantitative trait that contributes to NUtE, including nitrogen assimilation and remobilization efficiencies. The transcriptome profiles of the high-NUE cultivar K326 and the low-NUE cultivar TN90, combined with physiological data, suggested that the higher NUE of K326 was conferred by more advantageous changes in nitrogen uptake, nitrogen assimilation and transport, appropriate modulation of carbon and nitrogen metabolism, and maintenance of photosynthesis. Our findings will help elucidate the mechanisms underlying the response to nitrogen deficiency and improve plant NUE and yield in the field.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

Conception and design of the research: Yuqing Feng; acquisition of data: Yuanyuan Zhao and Xiaolong Chen; analysis and interpretation of data: Yanjun Ma; statistical analysis: Hongzhi Shi; drafting the manuscript: Yuqing Feng; revision of the manuscript for important intellectual content: Hongzhi Shi. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The Authors did not report any conflict of interest.

DATA AVAILABILITY STATEMENT

The raw sequence data reported in this study have been deposited in the Genome Sequence Archive (Chen et al., 2021) of the National Genomics Data Center (Yongbiao et al., 2022), China National Center for Bioinformation/Beijing Institute of Genomics, Chinese Academy of Sciences (GSA: CRA012572).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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