Received: 4 March 2023 | Revised: 31 August 2023 | Accepted: 20 September 2023

DOI: 10.1002/pld3.536

RESEARCH ARTICLE

Reducing nitrate and tobacco-specific nitrosamine level in burley tobacco leaves through grafting on flue-cured tobacco rootstock

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Funding information
The work was supported by the Shanghai Tobacco Corporation Research Program (grant number TP2019-C4). The funding source was not involved in the collection, analysis, or interpretation of data; in the writing of the report; or in the decision to submit the article for publication.

Abstract

Nitrosation of pyridine alkaloids in tobacco generates tobacco-specific nitrosamines (TSNAs), which are notable toxicants in tobacco products and smoke. Burley tobacco, a chloroplast- and nitrogen (N)-deficient phenotype that accumulates high levels of nitrate-nitrogen (NO_3-N) in its leaves, is particularly susceptible to TSNAs formation. In this study, reciprocal pot and field grafting experiments were conducted using burley tobacco Eyan No.1 and flue-cured tobacco K326 to investigate whether grafting burley tobacco scions on flue-cured tobacco rootstocks could enhance pigment biosynthesis and photosynthesis, while reducing the NO_3-N level in burley tobacco leaves. Grafting burley tobacco scions on flue-cured tobacco rootstocks significantly increased the total pigment content, photosynthetic rate, biomass, nitrate reductase and glutamine synthetase activities, as well as ammonium-nitrogen (NH_4-N), total soluble and reducing sugar, and soluble protein levels in burley tobacco leaves compared with burley tobacco self-rooting, while decreasing the NO_3-N level and nitrate-N to total N ratio. Transcriptomic analysis revealed that grafting resulted in upregulated expression of genes involved in starch, sucrose, porphyrin, chlorophyll, and N metabolism, as well as carbon fixation and carotenoid biosynthesis. The findings suggest that grafting on high N use efficiency rootstock is an exceptionally promising means of decreasing NO_3-N accumulation by improving photosynthesis and N metabolism in the scion, thereby reducing the levels of harmful TSNAs.

KEYWORDS
burley tobacco, flue-cured tobacco, grafting, NO_3-N content, TSNAs content

1 | INTRODUCTION

Tobacco (*Nicotiana tabacum* L.) is an economically important nonfood crop grown in several countries (Sierro et al., 2014). Cured leaves of different tobacco types (flue-cured, burley, and Oriental) are typically blended in the production of cigarettes. Burley tobacco is unique because it exhibits a chloroplast-deficient phenotype with impaired nitrogen (N) use efficiency (NUE) and reduced pigment content (Henika, 1932). Therefore, burley tobacco requires three to five times more N fertilization than flue-cured tobacco to achieve a comparable yield (Shang, 2007). Lewis et al. (2012) reported that reduced N absorption and utilization in burley tobacco are due to two homozygous recessive mutant alleles in the *yellow burley 1 (Yb1)* and *2 (Yb2)* loci. From the perspective of energy metabolism, increased nitrate

nitrogen (NO_3-N) accumulation in burley tobacco is due to its reduced levels of carbon assimilation, owing to a lower photosynthetic rate than that of other tobacco varieties (Li et al., 2018). Increased accumulation of NO_3-N in tobacco leaves is associated with the formation of tobacco-specific nitrosamines (TSNAs), representing a class of toxicants that contribute to cancer formation (Lewis et al., 2012). In burley tobacco, NO_3 is reduced to nitrite by microorganisms during leaf curing, which generates important nitrosating agents such as gaseous N oxide (NOx) during leaf storage (Wang et al., 2017). Therefore, decreasing the NO_3-N level in burley tobacco leaves is hypothesized to help decrease the risk of exposure to TSNAs in the population using tobacco products.

Photosynthesis, pigment content, nitrate reductase activity (NRA), glutamine synthetase activity (GSA), and total N (TN) level are highly related to NO_3-N level and should be considered when targeting NO_3-N reduction. In recent years, various strategies have been developed to reduce the NO_3-N level in burley tobacco leaves, including molecular modification (Lewis et al., 2012), chemical regulation (Li et al., 2018), and agronomic management (Sifola & Postiglione, 2003). However, many of these approaches are limited by chemical use regulations and agronomic management practices. Although the overexpression or knockout of key genes related to NO_3-N level can effectively alleviate its accumulation in burley tobacco leaves, these strategies are costly and can affect the quality of tobacco products. Moreover, the use of genetically modified organisms in agriculture is restricted (Bovet et al., 2022; Lu et al., 2016).

Grafting, an ancient cultivation technique, can effectively enhance plant resistance (Zhang et al., 2020), yield (Li et al., 2016), and photosynthesis (Liu et al., 2013) and reduce alkaloid synthesis (Ren et al., 2020). Notably, nutrient absorption in tobacco has been improved using cultivar rootstocks with high N absorption and NUE, thus alleviating NO_3-N accumulation (Ruiz et al., 2006). Additionally, grafting can positively alter the hormonal balance between the rootstock and scion (Albacete et al., 2015; Venema et al., 2017) and significantly enhance photosynthesis (Fullana-Pericàs et al., 2020). However, the effect of grafting on the NO_3-N level of burley tobacco leaves has not been reported. In our previous study, we determined that NUE and carbon assimilation differed significantly between flue-cured and burley tobacco, with flue-cured tobacco exhibiting a considerably higher NUE than burley tobacco (Li et al., 2017). Moreover, low pigment content, carbon fixation, and N assimilation were the main causes of abnormally high NO_3-N accumulation in burley tobacco.

Considering the above information, we hypothesized that grafting burley tobacco scions on flue-cured tobacco rootstock might improve photosynthesis and NUE in burley tobacco, thus decreasing the NO_3-N level in its leaves. Using a pot experiment and field trial, in this study, we investigated both physiological and transcriptomic changes to clarify whether the replacement of burley roots with flue-cured tobacco roots could decrease NO_3-N accumulation in burley tobacco leaves and elucidate the molecular mechanism underlying NO_3-N accumulation in burley tobacco.

2 | MATERIALS AND METHODS

2.1 | Plant material of pot experiment

A pot experiment was conducted with substrate cultures in a greenhouse located in the National Tobacco Cultivation & Physiology & Biochemistry Research Center of Henan Agricultural University (China) using the flue-cured K326 and burley Eyan No. 1 tobacco varieties. To ensure the success of grafting, tobacco seedlings were maintained without watering. The "split grafting" method was used when the seedlings developed six to eight true leaves. When new leaves emerged, the grafted plants were transplanted into plastic pots and cultivated in nutrient solution supplemented with Murashige and Skoog medium. The plants were grown at 25 ± 2°C under a 12/12 h light/dark cycle with an average photosynthetic photon flux density of 400 μmol m^{-2} s^{-1} and relative humidity of 65%–80%. The three graft groups were as follows: (1) K/K group, both scion and rootstock were K326; (2) E/E group, both scion and rootstock were Eyan No.1; and (3) E/K group, K326 was the rootstock and Eyan No.1 was the scion. The seeds of Eyan No.1 and K326 were provided by Hubei Tobacco Company and Henan Agricultural University, respectively. The collection of plant materials complied with the relevant institutional, national, and international guidelines and legislation.

2.2 | Plant material of field trial

The field trial was conducted in 2021 in Henan, China (30°43′N, 112°18′E). Topsoil (0–30 cm depth) properties were tested before transplanting. The soil in the field was mainly red soil with 10.06 g kg^{-1} organic matter, .83 g kg^{-1} TN, 7.32 mg kg^{-1} Olsen-phosphorus, 154.80 mg kg^{-1} Olsen-potassium, and pH 8.18. The materials, grafting method, and graft groups were identical to those used in the pot experiments. Plants in the graft groups were transplanted into the field using a randomized complete block design comprising 30 replicates. Nitrogen was applied at 45 and 180 kg ha^{-2} for flue-cured tobacco and burley tobacco, respectively.

2.3 | Sampling

In the pot experiment, leaf samples were collected for physiological and transcriptomic analyses 3 weeks after transplantation. Each treatment was performed on 18 uniform plants which were divided into two groups. The expanded leaves of 12 plants were frozen in liquid N to analyze the transcriptome, NRA, GSA, ammonium (NH_4)-N level, pigment content, and soluble protein level. Each stress treatment had three biological replicates, and every replicate contained four plants. The remaining plants were used to determine the photosynthetic rate, before being divided into roots, stems, and leaves for chemical analyses. The plant tissues were

heated at 105°C for approximately 15 min, dried to a constant weight at 65°C and then passed through a 60-mesh screen. The dried powder was analyzed for TN, NO_3-N, total soluble sugar, and reducing sugar level.

In the field trial, the net photosynthetic rate of plants subjected to each grafting combination was measured at 60 and 80 days after grafting. Pigment content and NO_3-N level were determined. When mature, the middle (positions 7–14) and upper (positions 15–20) leaves of adult tobacco plants were harvested and cured using a three-stage flue-curing process. TSNAs and NO_3-N levels in tobacco were determined after curing the leaves.

2.4 | Measurement of pigment content, net photosynthetic rate, and enzyme activities

The pigment content and NRA were determined using the methods described by Li (2000). GSA was tested according to the method described by O'Neal and Joy (1973). The net photosynthetic rate was measured between 9:00 and 11:00 am using the 6400XT Portable Photosynthesis System (LI-COR Biotechnology, Lincoln, NE, USA).

2.5 | Measurement of dry matter and carbonitride level

NO_3 level was determined using the method described by Cataldo et al. (1975). Leaf samples (∼1.0 g each) were frozen in liquid N and used to measure the soluble protein level according to the method described by Li (2000). Another set of leaf samples (∼.5 g each) was frozen in liquid N and used to measure the NH_4-N level according to the method described by Fan and Liang (1999). The levels of TN, total soluble sugar, and reducing sugar were determined according to methods modified from the Chinese Tobacco Industry standard (YC/T 161-2002 and YC/T 161, 159-2002). The levels of 4-(methylnitrosamino)-1-(3-pyridyl)-1-buta-none (NNN), (R,S)-N-nitrosoanatabine (NAT), (R,S)-N-nitrosoanabasine (NAB), and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) were determined using solid phase extraction liquid chromatography–tandem mass spectrometry as previously described (Sun et al., 2020). Total TSNAs were defined as the sum of NNN, NNK, NAT, and NAB.

2.6 | RNA extraction, preparation of cDNA libraries, and sequencing

The total RNA was isolated from plant leaves using the mirVana miRNA Isolation Kit (Ambion, Waltham, MA, USA). cDNA libraries were then synthesized using the TruSeq Stranded mRNA Library Prep Kit (Illumina, San Diego, CA, USA) and quantified and qualified using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Quality control was performed to produce clean data as previously described (Patel & Jain, 2012). Sequencing reads were mapped to the K326 tobacco reference genome (https://solgenomics.net/organism/Nicotiana_tabacum/genome) using TopHat software (Kim et al., 2013).

2.7 | Gene ontology (GO) term and Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment analyses of differentially expressed genes (DEGs)

Data normalization was performed based on read fragments per kilobase per million mapped reads, and the read counts of each gene were obtained using HTSeq-Count (Anders et al., 2015; Trapnell et al., 2012). DEGs were screened using DESeq (Anders & Huber, 2013). A p-adjust of <.05 and$|\log2(FC)| \geq 2$ was set as thresholds for significant differential gene expression. GO term and KEGG pathway enrichment analyses of DEGs were performed using the GOstats package v. 2.40.0 in R (http://bioconductor.org/packages/release/bioc/html/GOstats.html) (Falcon & Gentleman, 2007).

2.8 | Key gene expression validation using quantitative real-time polymerase chain reaction (qRT-PCR)

The expression of genes related to carbon and N metabolism obtained using RNA sequencing (RNA-Seq) was validated with qRT-PCR, which was performed on the 7900HT FAST Light Real-time PCR Instrument (Applied Biosystems, Waltham, MA, USA). The PCR mixtures were first incubated in a 384-well optical plate (Roche, Basel, Switzerland) at 50°C for 2 min, and then at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 60 s. The primers used for examining the expression of key genes are listed in Table 1.

TABLE 1 Primers used in qRT-PCR.

Gene symbol	Primer	Primer sequence (5′-3′)
CLH2	gene_30128-nF	TCCATCCGGAGTAGAGCCAA
	gene_30128-nR	CACAAGCTGGGAAGAGAGGG
NIR1	gene_84305-nF	GAGCTCCGGTTGACTGTTGA
	gene_84305-nR	CTCAATTATGGCTTGCCCGC
GLN1-1	gene_61180-nF	TTAACTGGCCTCTTGGCTGG
	gene_61180-nR	ACCGGCTGAGATTCCAACAG
RBCS	gene_35004-nF	TGGATGGGTTCCTTGCTTGG
	gene_35004-nR	CTCAGCCAAGACCTGAGTGG
Unknown	gene_75915-nF	GTGGTGGGGTTTGCTATGGA
	gene_75915-nR	ATGCTCACCTCCTACTGGGT

Abbreviation: qRT-PCR, quantitative real-time polymerase chain reaction.

2.9 | Statistical analysis

Regarding the chemical and physiological characteristics of the tobacco plants in the three graft groups, correlations and variance between groups were assessed using IBM SPSS Statistics v.20.0 (IBM Corp., Armonk, NY, USA). Groups were compared using the least significant difference multiple range test. All data correspond to the mean of three biological replicates ($n = 3$). And statistical analysis of the data was shown as means ± SD. Figures were obtained using GraphPad Prism v. 8..1 software (GraphPad Software Inc., San Diego, CA, USA).

Regarding gene expression, the correlation function (https://stat.ethz.ch/R-manual/Rdevel/library/stats/html/cor.html) in R v.3.4.1 (https://www.r-project.org) was used to calculate Pearson's correlation coefficient (*P*). The closer the *P* value is to 1, the higher the similarity in the expression patterns between samples. The prcomp function in R (https://stat.ethz.ch/R-manual/Rdevel/library/stats/html/prcomp.html) was used for dimension reduction in principal component analysis (PCA). The ggfortify package v..4.5 in R (https://mirrors.tuna.tsinghua.edu.cn/CRAN/bin/windows/contrib/3.4/ggfortify_0.4.5.zip) was used to construct the PCA diagram.

3 | RESULTS

3.1 | Differences in physiological characteristics and NO_3-N and TSNAs levels in the field trial

Chlorophyll *a* and *b* and carotenoid levels, total pigment content, and net photosynthetic rate were notably lower in the E/E group than in the K/K group, whereas the NO_3-N level was higher in the E/E group than in the K/K group (Figure 1a–f). The pigment content and photosynthetic rate were significantly higher in the E/K group than in the E/E group (Figure 1d,e). In contrast, the NO_3-N level was 50.37% and 55.95% lower in the E/K group than in the E/E group at 60 and 80 days after grafting, respectively. In addition, the NO_3-N level in air-cured leaves in the E/K group was 34.42% and 31.18% lower than that in the upper and middle leaves in the E/E group, respectively (Figure 1f). The NNN, NAT, NAB, NNK, and total TSNAs levels in the E/K group were reduced compared with those in the E/E group. The NNN, NAT, NAB, NNK, and total TSNAs levels in the upper leaves in the E/K group were 17.02%, 33.51%, 22.41%, 32.58%, and 18.87% lower than those in the upper leaves in the E/E group, respectively (Table 2). The NAT, NAB, and NNK levels in the middle leaves

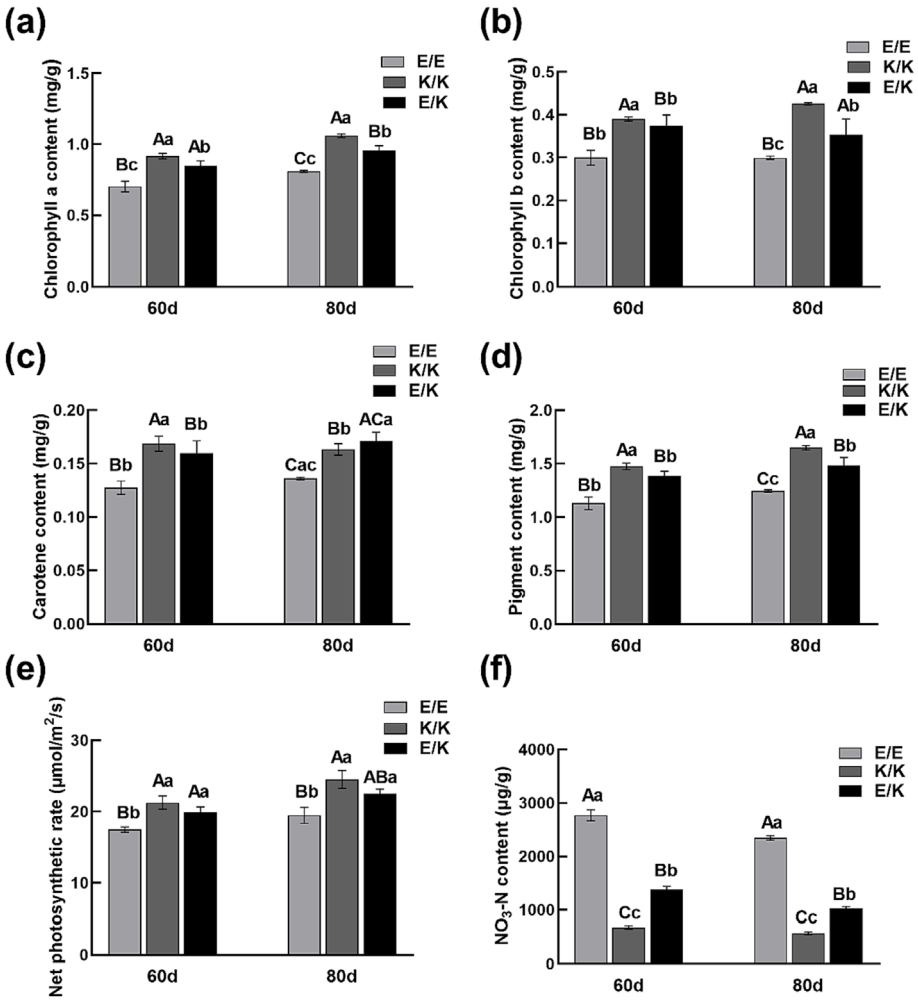

FIGURE 1 Differences in pigment content, net photosynthetic rate, and NO_3-N level in fresh tobacco leaves in field trial. (a) Chlorophyll *a* content. (b) Chlorophyll *b* content. (c) Carotenoid content. (d) Total pigment content. (e) Net photosynthetic rate. (f) NO_3-N level. K/K, both scion and rootstock were K326; E/E, both scion and rootstock were Eyan No.1; E/K, K326 was the rootstock and Eyan No.1 was the scion. Error bars indicate the standard error of the mean ($n = 3$ individuals per graft group). Uppercase and lowercase letters indicate significant differences between graft groups at $p < .01$ and $p < .05$, respectively.

TABLE 2 Effect of grafting on the NO_3-N and TSNAs levels in burley tobacco leaves.

Position	Graft group	NO_3-N (μg/g)	NNN (ng/g)	NAT (ng/g)	NAB (ng/g)	NNK (ng/g)	Total TSNAs (ng/g)
Upper leaf	K/K	445.95 ± 10.55Cc	81.40 ± 1.62Cc	89.95 ± 4.03Cc	4.68 ± .28Cc	23.00 ± .47Cc	199.03 ± 5.57Cc
	E/E	1833.35 ± 11.66Aa	8255.00 ± 41.59Aa	970.00 ± 6.51Aa	34.41 ± .92Aa	66.75 ± 1.37Aa	9326.16 ± 36.66Aa
	E/K	1202.27 ± 11.84Bb	6850.00 ± 164.59Bb	645.00 ± 11.62Bb	26.70 ± .41Bb	45.00 ± .93Cc	7566.70 ± 121.55Bb
Middle leaf	K/K	512.61 ± 7.63Cc	89.00 ± 1.65Cc	82.00 ± 4.82Cc	4.32 ± .08Cc	22.80 ± .33Cc	198.12 ± 6.52Cc
	E/E	2036.07 ± 58.03Aa	7685.00 ± 69.94Aa	1017.50 ± 5.05Aa	38.65 ± .66Aa	74.55 ± 1.47Aa	8815.70 ± 164.28Aa
	E/K	1401.26 ± 32.46Bb	7025.00 ± 82.59Bb	870.00 ± 21.09Bb	29.41 ± .39Bb	51.75 ± .80Bb	7976.16 ± 216.43Bb

Note: K/K, both scion and rootstock were K326; E/E, both scion and rootstock were Eyan No.1; E/K, K326 was the rootstock and Eyan No.1 was the scion. Uppercase and lowercase superscript letters indicate significant differences between graft groups at $p < .01$ and $p < .05$, respectively.

Abbreviations: NAB, (R,S)-N-nitrosoanabasine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NAT, (R,S)-N-nitrosoanatabine; NNN, 4-(methylnitrosamino)-1-(3-pyridyl)-1-buta-none; TSNAs, tobacco-specific nitrosamines.

in the E/K group were 14.50%, 23.91%, and 30.58% lower than those in the middle leaves in the E/E group, respectively (Table 2). The decrease in the TSNAs levels was mainly caused by a decrease in the NO_3-N level.

3.2 | Differences in pigment content and photosynthesis traits in the pot experiment

In the present study, the total pigment content represented the combined levels of chlorophyll *a* and *b* and carotenoids. The total pigment content, net photosynthetic rate, biomass accumulation, and carbohydrate level in the three graft groups are shown in Figure 2. The total pigment content and photosynthetic rate differed significantly among the graft groups. The chlorophyll *a* and *b* and carotenoid levels, as well as the total pigment content, were significantly lower in the E/E group than in the K/K group (Figure 2a–d). Furthermore, the net photosynthetic rate, biomass accumulation, and carbohydrate level were lower in the E/E group than in the K/K group (Figure 2e–h). The lower total pigment content may have influenced carbon fixation, leading to lower biomass and carbohydrate accumulation in the E/E group than in the K/K group. The total pigment content and photosynthetic rate were significantly improved in the E/K group. The chlorophyll *a* and *b* and carotenoid levels, total pigment content, and net photosynthetic rate increased by 20.17%, 22.43%, 48.38%, 25.44%, and 21.89% in the E/K group compared with those in the E/E group (Figure 2a–d). In addition, the E/K group had higher levels of total soluble and reducing sugars (21.05% and 79.44%, respectively) than the E/E group (Figure 2g,h). Irrespective of leaves, stems, or roots, biomass accumulation in the E/K group was higher than that in the E/E group. More N could be absorbed by the roots of flue-cured tobacco, thus increasing the total pigment content, which contributed to increased photosynthesis and carbohydrate accumulation.

3.3 | Differences in N compounds and enzyme activities related to N metabolism in the pot experiment

NRA, GSA, and N compounds were measured in tobacco seedling tissues, as shown in Figure 3. The NRA and GSA in the K/K group were 33.07% and 32.95% higher than those in the E/E group, respectively (Figure 3a,b). In addition, the NH_4-N and soluble protein levels were higher in the K/K group than in the E/E group, whereas the NO_3-N and TN levels and the NO_3-N to TN ratio showed the opposite trend (Figure 3c–g). However, N accumulation in the leaves, stems, and roots of the K/K group was higher than that in the E/E group (Figure 3h), which was consistent with the lower biomass accumulation in the E/E group. Grafting significantly influenced enzyme activities related to the nitrogen metabolism and accumulation of N compounds. The NRA, GSA, NH_4-N level, and soluble protein level in the E/K group increased by 13.29%, 14.58%, 16.06%,

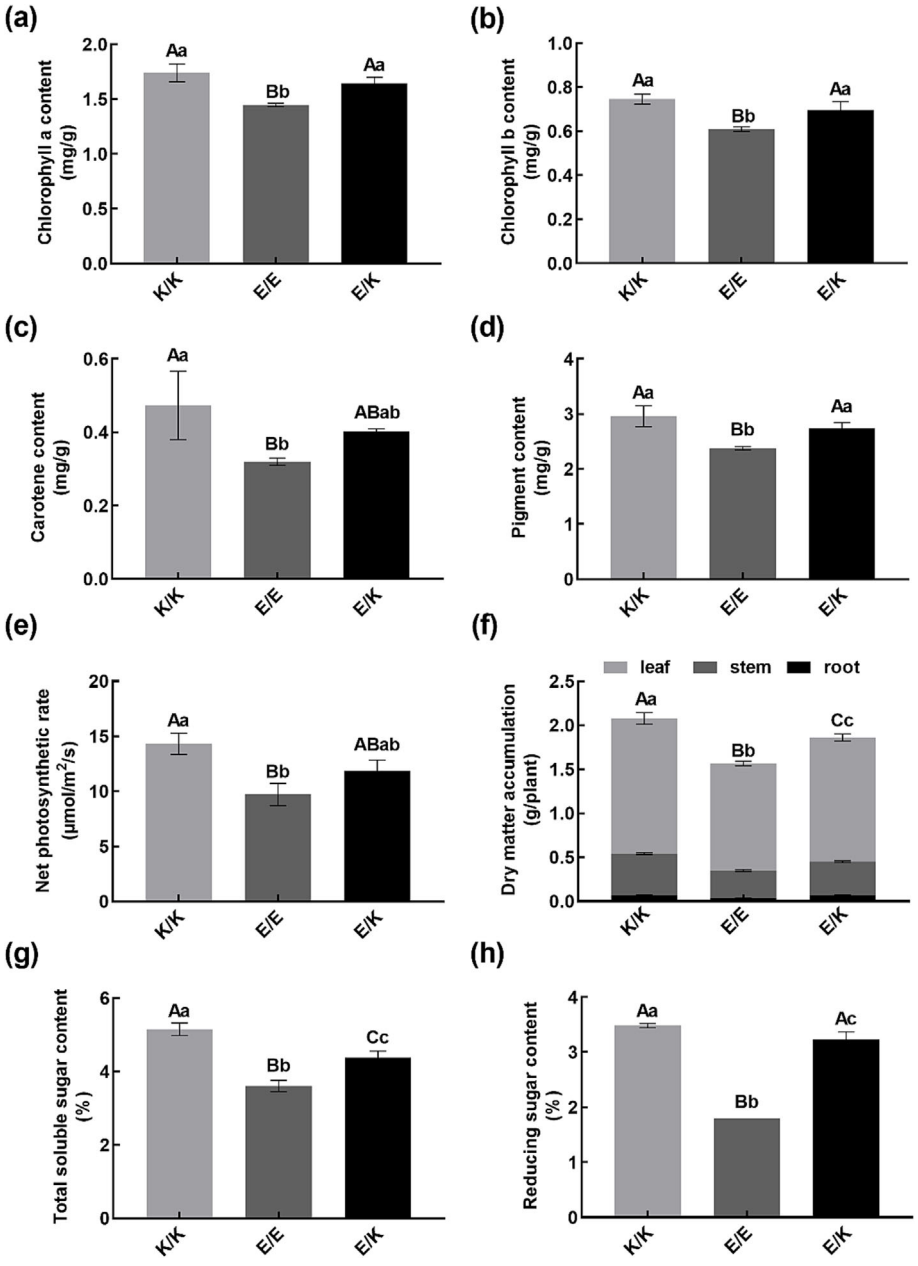

FIGURE 2 Differences in pigment content and photosynthesis traits in pot experiment. (a) Chlorophyll *a* content. (b) Chlorophyll *b* content. (c) Carotenoid content. (d) Total pigment content. (e) Net photosynthetic rate. (f) Biomass accumulation. (g) Total soluble sugar level. (h) Reducing sugar level. K/K, both scion and rootstock were K326; E/E, both scion and rootstock were Eyan no.1; E/K, K326 was the rootstock and Eyan No.1 was the scion. Error bars indicate the standard error of the mean ($n = 3$ individuals per graft group). Uppercase and lowercase letters indicate significant differences between graft groups at $p < .01$ and $p < .05$, respectively.

and 11.07% compared with those in the E/E group, respectively, whereas the NO_3-N level and NO_3-N to TN ratio decreased by 15.28% and 15.31%, respectively (Figure 3a–f). In addition, a significant increase in N accumulation was observed in the leaves, stems, and roots of the E/K group compared with that in the E/E group (Figure 3g,h).

3.4 | RNA-Seq results

Nine cDNA libraries were established for the RNA-Seq analysis. A total of 131.07, 128.42, and 128.62 million raw reads were identified in the K/K, E/E, and E/K groups, respectively. After data processing, 129.38 (98.71%), 126.30 (98.35%), and 126.79 (98.58%) million clean reads, respectively, were obtained for further analyses (Table S1). Over 94% of the clean reads across all samples were mapped to the tobacco reference genome using Hisat2 (Kim et al., 2015), most of which with unique locations in the genome. Over 92.05% of the sequencing data showed quality ≥30 (Q30), implying successful library construction and reliability for the subsequent analyses.

Gene expression levels were similar among samples from all three graft groups (Figure S1a). Furthermore, Pearson's correlation analysis and PCA indicated that the nine samples were highly correlated (Figure S1b,c). These analyses demonstrated that the sequencing data were representative and valid.

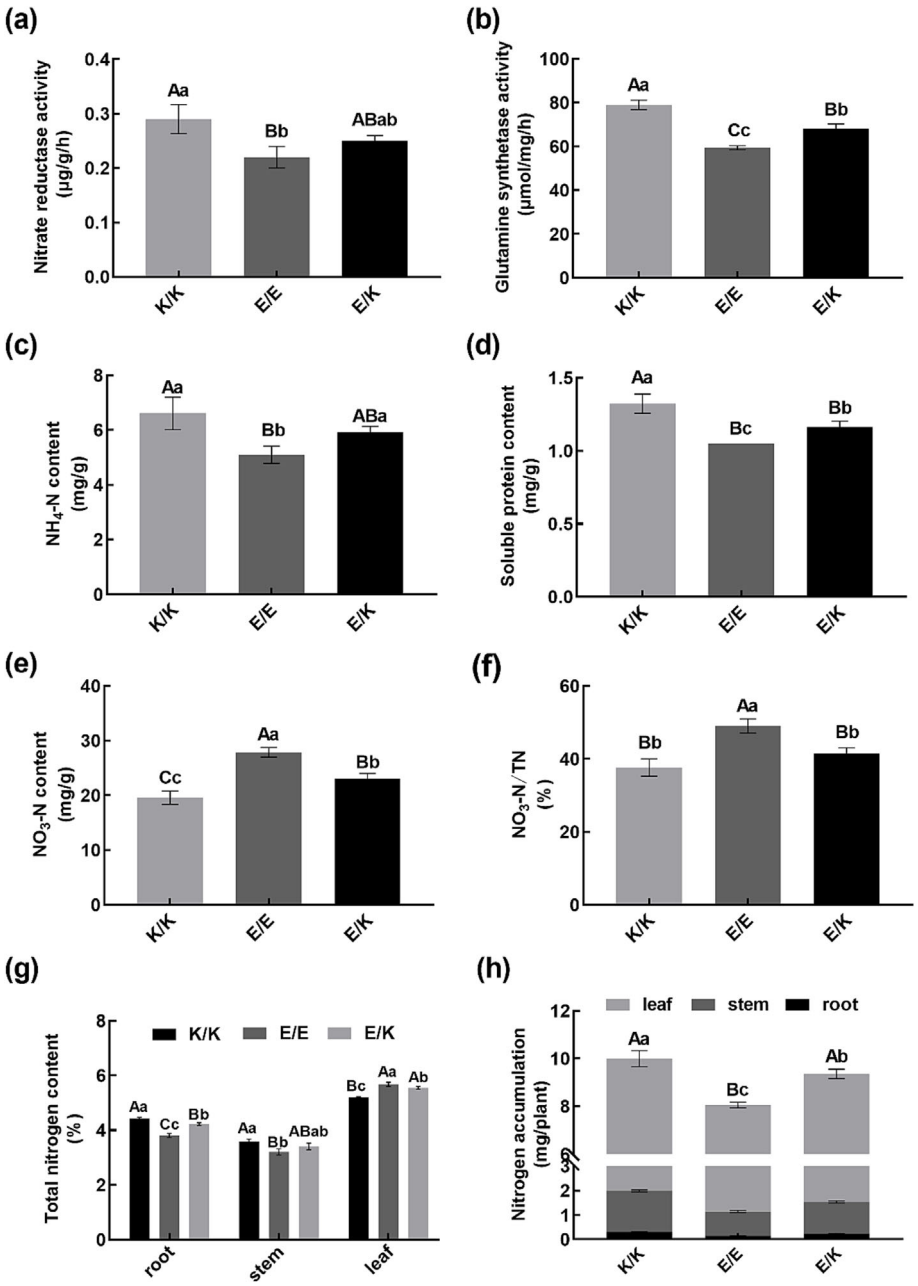

FIGURE 3 Differences in the activities of enzymes related to N metabolism and N compound level. (a) Nitrate reductase activity. (b) Glutamine synthetase activity. (c) NH_4-N level. (d) Soluble protein level. (e) NO_3-N level. (f) NO_3-N/TN ratio. (g) Total nitrogen level. (h) Nitrogen accumulation. K/K, both scion and rootstock were K326; E/E, both scion and rootstock were Eyan No.1; E/K, K326 was the rootstock and Eyan No.1 was the scion. Error bars indicate the standard error of the mean ($n = 3$ individuals per graft group). Uppercase and lowercase letters indicate significant differences between graft groups at $p < .01$ and $p < .05$, respectively.

3.5 | DEG selection and functional classification

A total of 8443 DEGs (4654 downregulated and 3789 upregulated) were obtained between the E/E and K/K groups, whereas 5777 DEGs (3472 downregulated and 2305 upregulated) were obtained between the E/K and E/E groups (Figure S2a). Hierarchical clustering analysis and volcano maps revealed that DEGs could distinguish samples of different graft groups (Figure S2b–S2d). In E/E-vs-K/K, downregulated DEGs were mainly enriched in terms such as pigment biosynthesis (GO:0046148), thylakoids (GO:0009579), photosystem II assembly (GO:0010207), photosystem I assembly (GO:0048564), photosystem II oxygen-evolving complex (GO:0009654), and starch metabolism (GO:0005982), whereas upregulated DEGs were mainly enriched in terms such as membrane (GO:0044425), plasma membrane (GO:0005886), and calcium ion binding (GO:0005509) (Figure 4a,b). In E/K-vs-E/E, downregulated DEGs were mainly enriched in terms such as phosphate-containing compound metabolism (GO:0006796), response to stimulus (GO:0050896), membrane (GO:0044425), and catalytic activity (GO:0003824), whereas upregulated DEGs were mainly enriched in terms such as chloroplast (GO:0044434), plastid (GO:0044435), and carbohydrate metabolism (GO:0005975) (Figure 4c,d). The KEGG pathway analysis indicated that DEGs involved in starch, sucrose, porphyrin, and chlorophyll metabolism, as well as carbon fixation and carotenoid biosynthesis, were

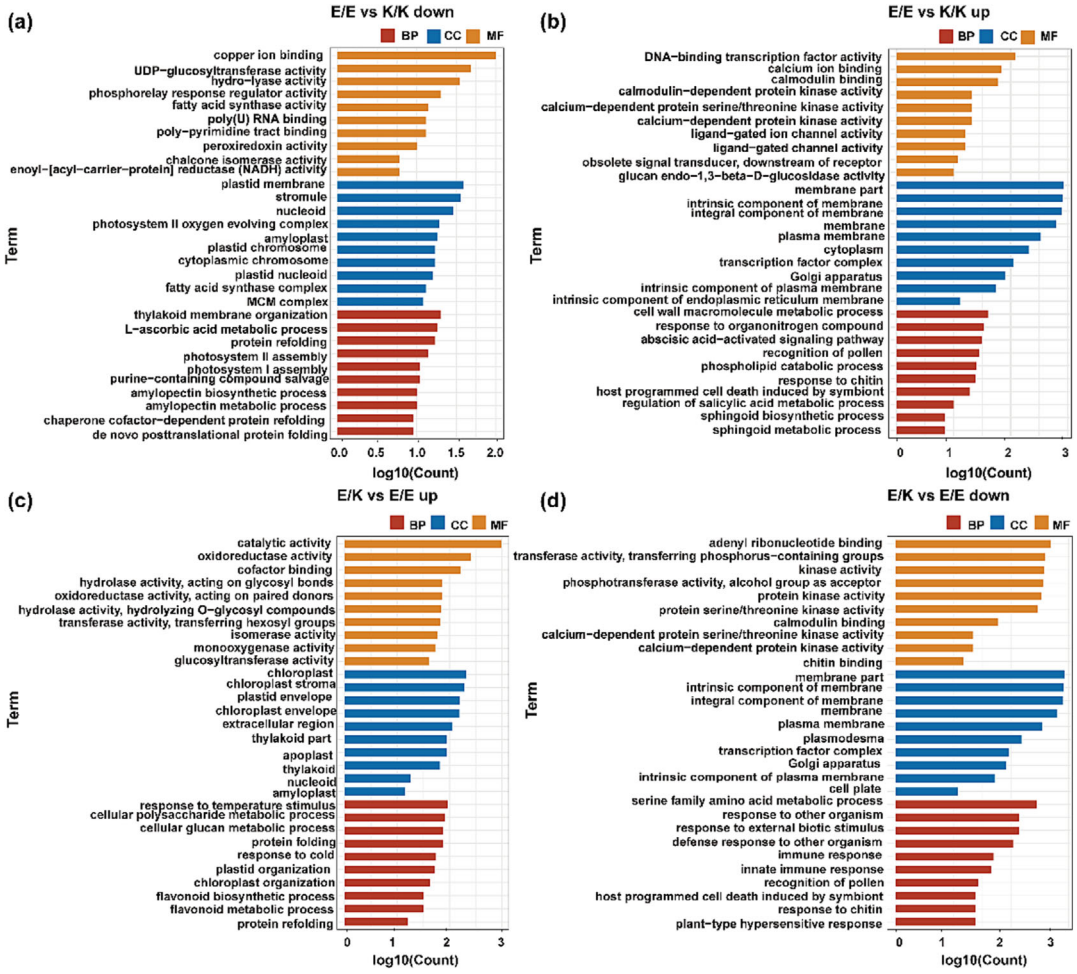

FIGURE 4 Gene ontology (GO) term enrichment of differentially expressed gene (DEG). (a) GO term enrichment of the downregulated DEGs between the E/E and K/K groups. (b) GO term enrichment of the upregulated DEGs between the E/E and K/K groups. (c) GO term enrichment of the upregulated DEGs between the E/K and E/E groups. (d) GO term enrichment of the downregulated DEGs between the E/K and E/E groups. K/K, both scion and rootstock were K326; E/E, both scion and rootstock were Eyan No.1; E/K, K326 was the rootstock and Eyan No.1 was the scion.

downregulated in E/E-vs-K/K and upregulated in E/K-vs-E/E (Figure 5a–d), which was consistent with the GO term enrichment results.

3.6 | GO term and KEGG pathway enrichment analyses of DEGs in response to grafting

To explore whether DEGs in the E/E group were enhanced by grafting, Venn diagram analysis of upregulated DEGs between the E/K and E/E groups and downregulated DEGs between the E/E and K/K groups was performed (Figure 6a). The GO term and KEGG pathway enrichment analyses were then conducted to examine the potential functions and metabolic pathways of the enriched DEGs. The Venn diagram revealed that 1590 DEGs were enriched among the upregulated DEGs between the E/K and E/E groups and downregulated DEGs between the E/E and K/K groups. According to the GO term and KEGG pathway enrichment analyses (Figure 6b,c), these DEGs were significantly enriched in pathways associated with starch, sucrose, porphyrin, and chlorophyll metabolism, carbon fixation, and carotenoid biosynthesis.

3.7 | DEGs involved in carbon and nitrogen metabolism

Transcriptome sequencing technology provides solid information on DEGs involved in specific biological responses. To further understand how the expression of nitrogen and carbon metabolism-related genes in the E/K group changed compared with that in the E/E group, the DEGs involved in starch, sucrose, porphyrin, chlorophyll, and N metabolism, carbon fixation, and carotenoid biosynthesis were further investigated (Figure 7). The results indicated that these DEGs were downregulated in the E/E group and upregulated in the E/K group compared with those in the K/K group, which might explain the lower NO_3-N level in the E/K group than in the other two groups. Supporting information Tables S2–S6 provide information on metabolic pathways and DEGs.

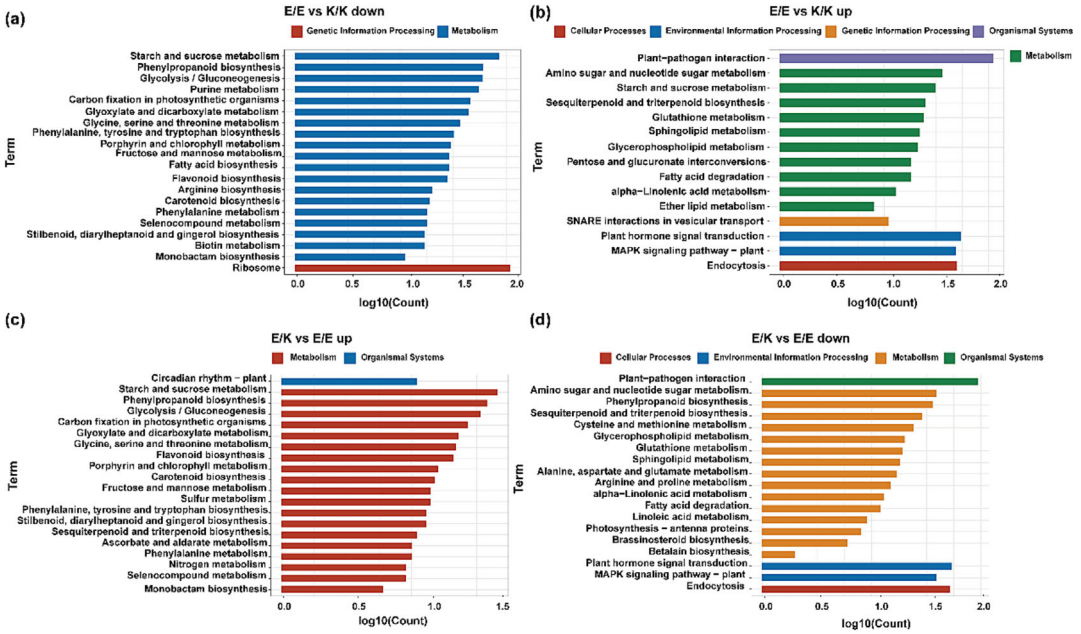

FIGURE 5 Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis of differentially expressed gene (DEG). (a) KEGG pathway analysis of the downregulated DEGs between the E/E and K/K groups. (b) KEGG pathway analysis of the upregulated DEGs between the E/E and K/K groups. (c) KEGG pathway analysis of the upregulated DEGs between the E/K and E/E groups. (d) KEGG pathway analysis of the downregulated DEGs between the E/K and E/E groups. K/K, both scion and rootstock were K326; E/E, both scion and rootstock were Eyan No.1; E/K, K326 was the rootstock and Eyan No.1 was the scion.

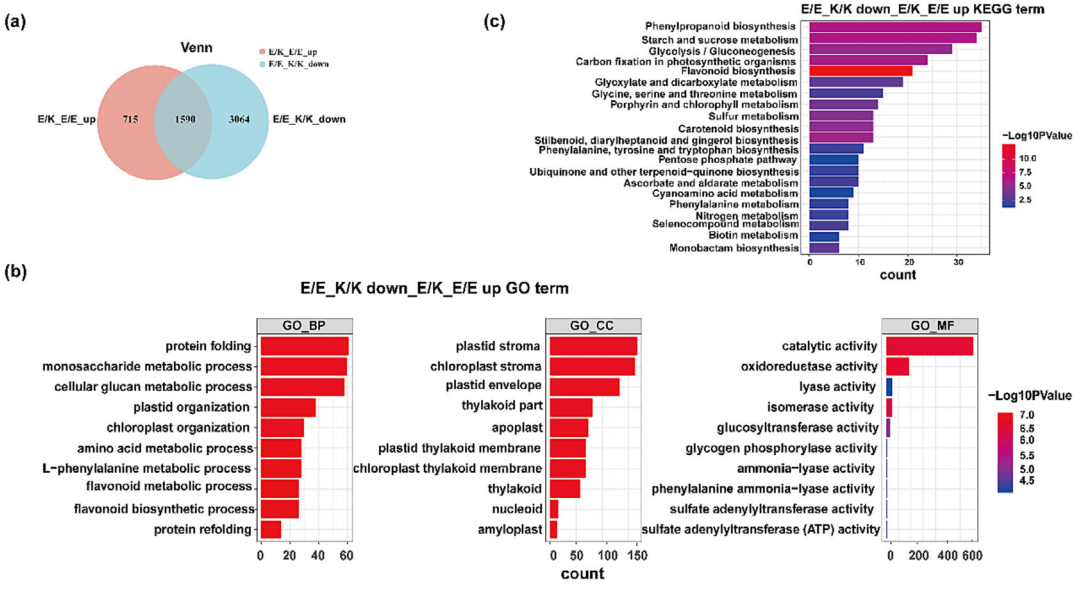

FIGURE 6 Gene ontology (GO) term and Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment analyses of differentially expressed genes (DEGs) in response to grafting. (a) Venn diagram of downregulated DEGs between the E/E and K/K groups (E/E_K/K down) and upregulated DEGs between the E/K and E/E groups (E/K_E/E up). (b) GO term enrichment by DEGs in response to grafting. (c) Enriched KEGG pathways by DEGs in response to grafting. K/K, both scion and rootstock were K326; E/E, both scion and rootstock were Eyan No.1; E/K, K326 was the rootstock and Eyan No.1 was the scion.

3.8 | Validation using qRT-PCR

Key genes related to carbon and nitrogen metabolism were selected for qRT-PCR analysis. Among them, *gene_30128(CLH2)*, *gene_84305* (*NIR1*), *gene_61180* (*GLN1−1*), *gene_35004* (*RBCS*), and *gene_79195* (unknown) were upregulated in the E/K group compared with those in the E/E group (Figure 8). The qRT-PCR results were highly consistent with those detected using transcriptome sequencing, confirming the reliability of the RNA-Seq results.

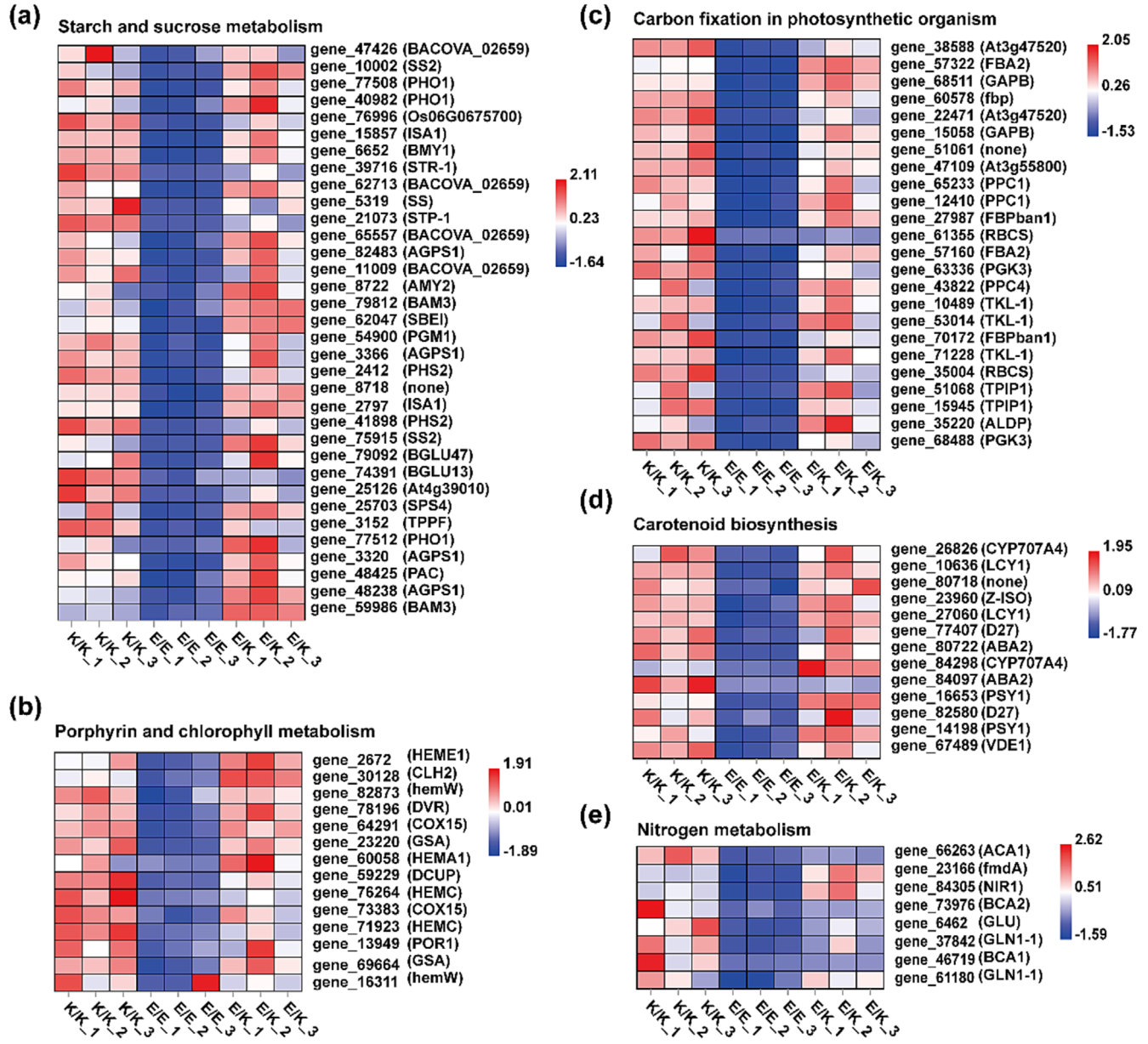

FIGURE 7 Differentially expressed genes (DEGs) involved in carbon and N metabolism. (a) DEGs involved in starch and sucrose metabolism. (b) DEGs involved in porphyrin and chlorophyll metabolism. (c) DEGs involved in carbon fixation. (d) DEGs involved in carotenoid biosynthesis. (e) DEGs involved in N metabolism. K/K, both scion and rootstock were K326; E/E, both scion and rootstock were Eyan No.1; E/K, K326 was the rootstock and Eyan No.1 was the scion.

4 | DISCUSSION

The growth and yield of N-efficient genotypes are higher than those of N-inefficient genotypes under normal or low-nitrogen conditions (Mansour et al., 2017; Zhang et al., 2018). Previous studies by Lewis et al. (2012) and Li et al. (2017) indicated that burley tobacco has weak NUE and N assimilation capacities, leading to lower biomass and higher NO_3-N accumulation than those observed in other tobacco types. In the present study, burley tobacco grafted on flue-cured tobacco (E/K) presented low NO_3-N accumulation in leaves (Figure 3e). Additionally, the root, stem, and leaf biomasses were lower in self-grafted burley tobacco (E/E) than in self-grafted flue-cured tobacco (K/K) (Figure 2f). However, grafting burley tobacco on flue-cured tobacco (E/K) improved the growth of burley tobacco, indicating the rootstock had a significant influence on scion growth. These results are in line with those reported by Nawaz et al. (2018), that is, grafting on rootstock from wild watermelon with high NUE substantially improved whole plant growth.

Chlorophyll content is used as an indicator of photosynthetic performance, which is needed to provide the energy and carbon skeleton for plant growth and N assimilation (Lewis et al., 2012; Nunes-Nesi et al., 2010). Burley tobacco is a chloroplast-deficient mutant with

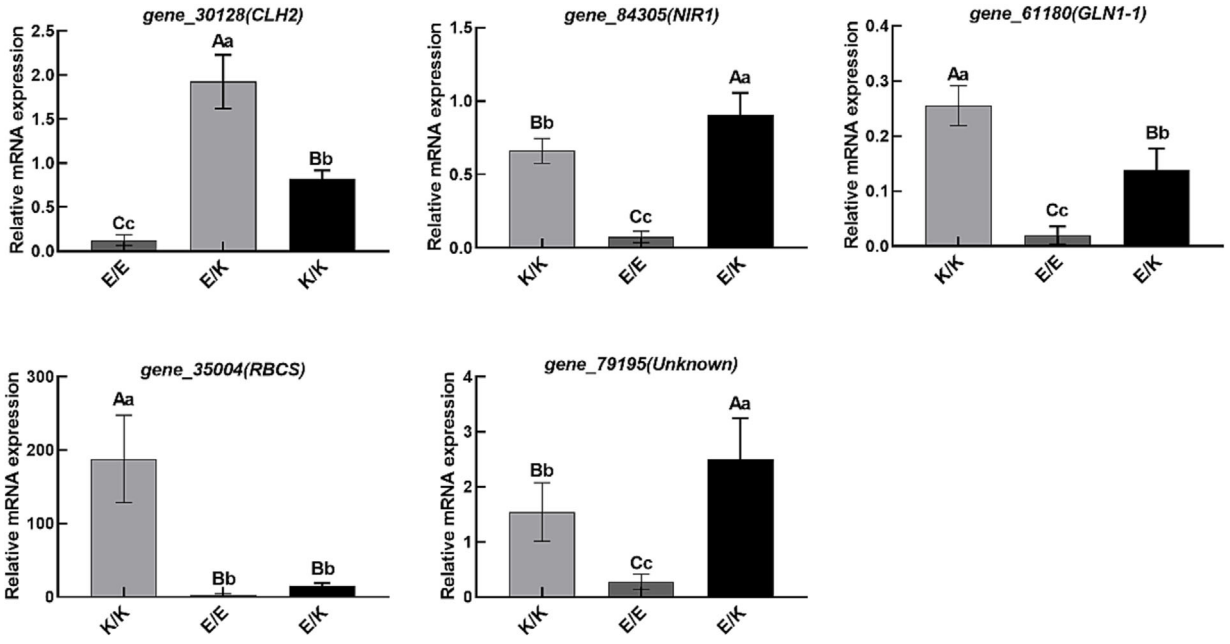

FIGURE 8 Gene expression levels obtained using qRT-PCR. K/K, both scion and rootstock were K326; E/E, both scion and rootstock were Eyan No.1; E/K, K326 was the rootstock and Eyan No.1 was the scion. Error bars represent standard error of the mean ($n = 3$ individuals per graft group). Uppercase and lowercase letters indicate significant differences between the graft groups at $p < .01$ and $p < .05$, respectively.

reduced pigment content and photosynthetic capacity (Henika, 1932). In the present study, grafting burley tobacco on flue-cured tobacco enhanced the total pigment content, net photosynthetic rate, and photosynthesis products of burley tobacco (Figure 2a–h); increased TN level; and promoted N accumulation in the root (Figure 3g,h). This finding suggests that flue-cured tobacco rootstocks have an increased nutrient acquisition capacity, which translates into high leaf chlorophyll content and photosynthetic production (Oda et al., 2005; Rouphael et al., 2008). These results are similar to those obtained for other field crops such as tomatoes, in which an increase in the absorption and utilization of N and photosynthetic rate was observed in grafted plants (Fullana-Pericàs et al., 2020; Zhang et al., 2021). Studies have also shown that grafting substantially affects the endogenous hormone level in plant leaves, exerting a regulatory effect on their photosynthetic capacity (Zhou et al., 2007, 2009), although this was not examined in our study. Furthermore, transcriptomic analysis revealed 5777 DEGs between the E/K and E/E groups; these genes were significantly enriched in pathways associated with starch, sucrose, porphyrin, chlorophyll, and nitrogen metabolism; carotenoid biosynthesis; and carbon fixation (Figures S2a and 5c,d). Further investigation of downregulated DEGs between the E/E and K/K groups and upregulated DEGs between the E/K and E/E groups suggested that the flue-cured tobacco rootstock promoted pigment biosynthesis and photosynthesis in burley tobacco leaves (Figure 6a–c). Genes encoding protochlorophyllide reductase *(POR, gene_13949)*, porphobilinogen deaminase *(PBG, gene_76264)*, and glutamate-1-semialdehyde 2,1-aminomutase *(GSA, gene_69664)* were substantially upregulated by grafting burley tobacco on flue-cured tobacco (Figure 7b). Furthermore, genes related to carbon fixation and starch and sucrose metabolism were upregulated by grafting burley tobacco onto flue-cured tobacco (Figure 7a,c). These genes included sedoheptulose-1,7-bisphosphatase *(SBP, gene_47109)*, which encodes a key enzyme in ribulose-1,5-bisphosphate (RuBP) regeneration and Calvin cycle process [41], and fructose-bisphosphate aldolase *(FBA, gene_57322)*, which has a major influence on the growth and photosynthesis of tobacco (Lefebvre et al., 2015; Uematsu et al., 2012).

Nitrogen metabolism requires energy and a carbon skeleton generated during photosynthesis and carbon fixation (Lewis et al., 2012; Nunes-Nesi et al., 2010). In the present study, the NO_3-N level and NO_3-N to TN ratio in burley tobacco were significantly decreased using flue-cured tobacco as the rootstock, indicating that the replacement of the root system reversed the disadvantage of the scion. Nitrogen metabolism is one of the most important and basic metabolic pathways in plants, and NO_3 level is highly correlated with the capacity for N assimilation (Li et al., 2017; Wang et al., 2019). Nitrate reductase, nitrite reductase (NiR), and GS play a key role in N assimilation (Thomsen et al., 2014; Yu et al., 2021). Previous studies have indicated that grafting improves tomato yield by increasing GSA (Kim et al., 2013). Our results demonstrated that grafting burley tobacco on flue-cured tobacco increased NRA and GSA in burley tobacco (Figure 3a,b). The higher efficiency of NO_3-N reduction and assimilation in grafted plants was confirmed by higher levels of NH_4-N, protein, and TN in burley tobacco grafted on flue-cured tobacco than in self-grafted burley tobacco (Figure 3c,d,g). Moreover, the expression of *NiR (gene_84305)* and *GS (gene_61180)* was significantly upregulated, indicating that nitrogen metabolism was markedly stimulated in grafted burley tobacco with a flue-cured tobacco rootstock (Figure 8).

In conclusion, the study findings demonstrated that NO_3-N reduction in burley tobacco was related to a significant increase in total pigment content, photosynthetic rate, and carbohydrate and nitrogen assimilation capacity, indicating that rootstocks from plants with better NUE can be used in grafting to improve this characteristic in less efficient plants. Grafting of tobacco plants can be defined as a rapid and effective alternative to enhance pigment biosynthesis and photosynthesis, thus increasing tobacco yield while decreasing NO_3-N level and the subsequent accumulation of carcinogenic TSNAs in tobacco leaves.

AUTHOR CONTRIBUTIONS

H. S. and Y. F. conceived and designed the study. Y. F., G. L., and Y. Z. conducted the study. Y. F., G. L., and Y. Z. analyzed the data and prepared Figures 1–8. Y. F. drafted the manuscript. H. S. completed the manuscript revision for important intellectual content. All authors contributed to article preparation and approved the submitted version.

ACKNOWLEDGMENTS

We would like to thank Editage (www.editage.cn) for English language editing.

CONFLICT OF INTEREST STATEMENT

The Authors did not report any conflict of interest.

PEER REVIEW

The peer review history for this article is available in the Supporting Information for this article.

DATA AVAILABILITY STATEMENT

Sequencing data have been deposited in the National Center of Biotechnology Information database (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA720751).

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SUPPORTING INFORMATION

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

How to cite this article: Feng, Y., Zhao, Y., Li, G., & Shi, H. (2023). Reducing nitrate and tobacco-specific nitrosamine level in burley tobacco leaves through grafting on flue-cured tobacco rootstock. *Plant Direct*, *7*(10), e536. https://doi.org/10.1002/pld3.536