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Integrated Transcriptomic and Metabolomic Analyses Reveal the Synergistic Regulatory Mechanisms of Nitrogen Application Rate and Topping on Tobacco Leaf Quality

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Abstract

Nitrogen application rate and topping are two key agronomic factors affecting tobacco leaf quality, but the synergistic regulatory mechanisms remain unclear. This study aimed to elucidate the physiological and molecular mechanisms by which nitrogen application rate and topping synergistically regulate tobacco leaf quality, and to identify key candidate genes involved in nicotine biosynthesis. Using the tobacco cultivar K326, three nitrogen application levels were established: low (0 kg/ha), medium (120 kg/ha), and high (210 kg/ha). Agronomic traits and rhizosphere soil physicochemical properties were monitored, carbon and nitrogen metabolism enzyme activities were measured before and after topping, and mature leaf chemical composition was determined, integrated with transcriptomic and metabolomic analyses. The results showed that medium nitrogen achieved high yield while enhancing organic matter turnover efficiency, reducing rhizosphere water-soluble chloride content, and alleviating soil acidification and ammonium–potassium ion uptake competition caused by excessive fertilization. Combined with topping, it significantly enhanced carbon and nitrogen metabolism, promoted starch-to-soluble-sugar conversion, and increased nicotine accumulation, thereby optimizing leaf chemical composition. Integrated transcriptomic and metabolomic analyses revealed that medium nitrogen combined with topping exerted the most pronounced effects on gene expression and metabolite accumulation. Weighted gene co-expression network analysis (WGCNAs) identified a module highly correlated with nicotine biosynthesis, with hub genes mainly annotated as cytochrome P450 and terpene synthase family members. In conclusion, this study elucidated the molecular mechanisms by which the combination of medium nitrogen application and topping, as an integrated agronomic practice, improves tobacco leaf quality through coordinating carbon-nitrogen metabolism and optimizing chemical composition, and identified key candidate genes potentially regulating nicotine biosynthesis, providing a theoretical basis for nitrogen fertilizer management and high-quality cultivation of tobacco.

Keywords: nitrogen application rate; topping; leaf quality; carbon and nitrogen metabolism

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1. Introduction

Tobacco (*Nicotiana tabacum* L.) is an important economic crop cultivated worldwide. According to statistics from the Food and Agriculture Organization, global tobacco production reached approximately 6.048 million tonnes in 2024 (https://ourworldindata.org/grapher/tobacco-production?time=latest, accessed on 17 October 2026). Major producing countries include China, India, Brazil, and the United States, endowing tobacco with significant economic value [1]. Leaf quality is a critical factor influencing its economic value. Tobacco leaf quality is a comprehensive concept that refers to the overall characteristics exhibited during use. It is influenced by numerous factors, including the sugar-to-alkaloid ratio, potassium-to-chloride ratio, phenols, and terpenoids [2]. Nicotine is the most abundant alkaloid in tobacco leaves, typically accounting for over 90% of total alkaloids; it directly affects leaf quality and usability and is the main addictive component driving tobacco product consumption [3]. Glucose and fructose, classified as reducing sugars, primarily improve tobacco taste by balancing the sensory effects of other alkaloids [4]. Consequently, tobacco leaves with high reducing sugar content are preferred for tobacco products [5], and the potassium-to-chloride ratio mainly improves flavor, enhances combustion quality, and contributes to overall leaf quality [6].

Nitrogen is an essential macronutrient and a fundamental constituent of nucleic acids, proteins, chlorophyll, alkaloids, vitamins, and phytohormones, and it plays an indispensable role in plant growth and development [7]. In commercial tobacco production, substantial nitrogen fertilization is typically required to achieve optimal yield and quality, which often results in excessive nitrogen application [8]. However, excessive fertilization can induce soil degradation, manifesting as soil compaction, reduction in organic matter content, disruption of soil nutrient homeostasis, and even soil acidification [9]. Tobacco plants generally rely on high rates of nitrate (NO_3^-) uptake from the soil to sustain growth and ensure quality. Nitrate enters the plant through root-localized nitrate transporters (NRTs), with only a minor fraction assimilated in the roots [10]; the majority is translocated to the leaves, where nitrate reductase (NR) and nitrite reductase (NiR), localized in the cytosol and plastids, respectively, sequentially reduce it to ammonium (NH_4^+). The resulting ammonium is then incorporated into glutamine (Gln) and glutamate (Glu) via the glutamine synthetase (GS) glutamate synthase (GOGAT) pathway [11]. Previous studies have shown that overexpression of the nitrate-transporter-encoding gene *NtNRT2.11* enhances nitrogen use efficiency, biomass accumulation, and potassium (K^+) content in tobacco [12].

Nicotine, one of the most critical indicators of tobacco leaf quality, is predominantly synthesized in the roots, subsequently translocated to the leaves via the xylem, and sequestered in vacuoles. Insect herbivory and physical damage have been shown to increase nicotine accumulation. The nicotine content in tobacco is modulated by multiple factors, including cultivation practices and environmental conditions [13]. As a nitrogen-containing and typically alkaline compound, nicotine is composed of a pyrrolidine ring and a pyridine ring, and its biosynthesis proceeds mainly from amino acids and their derivatives, catalyzed by a series of enzymes [14]. Consequently, the nitrogen application rate significantly influences nicotine content. To date, the complete biosynthetic pathway of nicotine and the enzymes responsible for it have been fully characterized [15].

Topping is a conventional agronomic practice in which the apical inflorescence and a portion of the upper young leaves are removed when tobacco plants reach the budding stage [16]. At this stage, tobacco transitions from vegetative to reproductive growth, and nutrients are redirected toward the apical floral meristems. This shift is detrimental to leaf development, biomass accumulation, and the balance of endogenous chemical constituents, ultimately compromising leaf quality. Topping significantly affects carbon and nitrogen metabolism in tobacco by eliminating apical dominance, redistributing nutrients,

enhancing dry matter accumulation, and promoting leaf senescence and ripening, thereby improving tobacco leaf quality [17,18]. Furthermore, topping influences source–sink relationships, hormonal homeostasis, root system development, and secondary metabolism in tobacco [19].

Although considerable research has examined the regulation of nitrogen application rates and topping as individual practices, systematic investigations into the synergistic mechanisms through which these two practices jointly modulate tobacco leaf quality remain limited. Therefore, we hypothesized that an optimal nitrogen application rate exists that can balance vegetative growth and leaf quality, and that topping under this nitrogen regime would better coordinate leaf chemical composition, thereby improving quality. Accordingly, our objectives were to (1) evaluate the effects of different nitrogen application rates on agronomic traits, leaf quality, and rhizosphere soil physicochemical properties across growth stages; (2) elucidate the physiological and molecular responses of carbon and nitrogen metabolism to topping under different nitrogen application rates; (3) identify key genes involved in nicotine biosynthesis through integrated transcriptomic and metabolomic analyses; and (4) elucidate the molecular mechanisms by which nitrogen application rate and topping synergistically regulate tobacco leaf quality. Given the economic importance of tobacco, our findings not only deepen the fundamental understanding of carbon–nitrogen metabolism coordination under agronomic interventions but also provide a theoretical basis for optimizing nitrogen fertilizer management in tobacco production, with potential application value for sustainable agricultural practices, improved nitrogen use efficiency, and quality-oriented cultivation strategies. Collectively, these results provide a theoretical foundation and technical support for nitrogen fertilizer management and high-quality cultivation practices in tobacco production.

2. Materials and Methods

2.1. Plant Materials, Growth Conditions, and Treatments

The tobacco cultivar K326 used in this study was provided by the Chuxiong Prefecture Company of Yunnan Tobacco Company, China. The experimental site (which had been continuously cropped for 4 years prior to the experiment) is a typical tobacco-growing area in Yunnan Province: Donghua Town, Chuxiong City, Chuxiong Yi Autonomous Prefecture (24°58′ N, 101°28′ E), with an altitude of 1803.9 m. The planting period in 2024 was from April to August, with seedling raising in April and transplanting in May. The average temperatures from April to August were 28, 27, 27, 27, and 28 °C, respectively, and the average precipitation was 0.3, 3.05, 4.78, 6.75, and 5.4 mm, respectively. According to the Chinese Soil Classification System, the soil in Donghua Town, Chuxiong City, is purple soil. Before planting, the soil pH was 5.83, with soil organic matter of 29.8 g/kg, alkaline hydrolyzable nitrogen of 186 mg/kg, available phosphorus of 39.3 mg/kg, and available potassium of 211 mg/kg.

To elucidate the molecular and physiological mechanisms by which nitrogen application rate and topping affect tobacco quality, three different nitrogen applications were established: low nitrogen (0 kg/ha pure N), medium nitrogen (120 kg/ha pure N), and high nitrogen (210 kg/ha pure N). Each nitrogen concentration consisted of three replicate plots (each plot approximately 23 m^2, with a total area of approximately 207 m^2), with 35 plants per plot and a planting density of 16,725 plants/ha (row spacing of 1.2 m, plant spacing of approximately 60 cm). Protective rows were set around the perimeter. Tobacco seedlings were raised using the floating seedling method, and healthy seedlings with 4–5 leaves and a height of 5–7 cm were selected for transplanting. The basal fertilizer used was a special compound fertilizer for flue-cured tobacco (N-P_2O_5-K_2O = 15-15-18), and the topdressing fertilizer was a nitrogen–potassium fertilizer (N-P_2O_5-K_2O = 12.5-0-33.5). The basal fertil-

izer was applied via hole application (placed into the planting holes), while the topdressing was applied by dissolving the fertilizer in water and directly applying it to the root zone of the tobacco plants (root drenching/fertigation). The low-nitrogen treatment received no fertilizer. For the medium-nitrogen treatment, the basal fertilizer application rate was 480 kg/ha, with topdressing applied once each at the rosette and vigorous growing stages at 192 kg/ha per application. For the high-nitrogen treatment, the basal fertilizer application rate was 840 kg/ha, with topdressing applied once each at the rosette and vigorous growing stages at 336 kg/ha per application. Agronomic traits and soil physicochemical properties were systematically monitored across different growth stages of tobacco. Key enzyme activities related to carbon and nitrogen metabolism, as well as transcriptomic and metabolomic analyses, were measured before and after topping under different nitrogen concentrations, and a comprehensive evaluation of the quality of mature tobacco leaves was conducted.

2.2. Soil Sampling and Physicochemical Property Determination

Rhizosphere soil samples were collected at the rosette (week 5), vigorous growing (week 11), and budding (week 14) stages under three different nitrogen concentration treatments. Using an "S"-shaped sampling method, plants were uprooted and large clods of loose soil were gently shaken off. The soil tightly adhering to the root surface (0–2 mm) was brushed off and collected as rhizosphere soil. Three plants from each plot were pooled as one biological replicate. After removing fine roots and gravel, the pH, soil organic matter (SOM), total nitrogen (TN), alkaline hydrolyzable nitrogen (AN), available phosphorus (AP), available potassium (AK), and water-soluble chloride (WSC) of the rhizosphere soil were determined according to the Chinese agricultural and forestry industry standards NY/T 1377-2007, NY/T 1121.6-2006, LY/T 1228-2015, NY/T 1121.7-2014, NY/T 889-2004, and NY/T 1378-2007, respectively. The above was performed with reference to Liao et al. [20]).

2.3. Investigation of Agronomic Traits and Quality of Tobaccos

Agronomic traits, including plant height, stem girth, number of effective leaves, internode length, and length and width of the fifth leaf, were measured at the rosette, vigorous growth, and budding stages under the three nitrogen application rates. All measurements were conducted according to YC/T 142-2010 using a random sampling method. For each nitrogen treatment, three plants were selected from each of the three replicate plots, totaling nine plants per treatment. At the budding stage, topping was performed when plants reached 95–110 cm in height, and 18–20 leaves were retained per plant. The 9th mature leaves from the top (middle leaves) of the tobacco plants were harvested and flue-cured according to the Chinese National Standard GB/T 23219-2008. The contents of total sugar and reducing sugar in the cured leaves were determined according to YC/T 159-2002, while those of total alkaloids, total nitrogen, potassium, and chloride were determined according to YC/T 160-2002, YC/T 161-2002, YC/T 173-2002, and YC/T 162-2002, respectively. The above was performed with reference to Liao et al. [21].

2.4. Measurement of Key Carbon and Nitrogen Metabolism Indicators After Topping

At the budding stage, topping was performed at a plant height of 95–110 cm, and 18–20 leaves were retained per plant. The 9th leaves from the top (middle leaves) were collected under the three nitrogen treatments before topping and 10 days after topping to determine carbon- and nitrogen-metabolism-related enzyme activities, as well as the contents of nicotine, free ammonia, sucrose, starch, and total sugar. Three biological replicates were set for each treatment.

Nitrate reductase (NR) and glutamine synthetase (GS) activities in tobacco leaves were determined as described in reference [22]. Tobacco leaves were collected and cut into 0.5 cm^2 segments. A 0.5 g aliquot of leaf segments was placed into test tubes. To each tube, 9 mL each of potassium nitrate, isopropanol, and phosphate buffer were added, and one tube was supplemented with trichloroacetic acid (TCA) to serve as the blank control. The remaining tubes were incubated at 30 °C for 30 min, after which TCA was added to terminate the enzyme reaction, followed by vortexing to ensure thorough mixing. After standing for 2 min, a 1 mL aliquot of the supernatant was transferred to a new test tube, to which 2 mL of sulfanilamide reagent and 2 mL of α-naphthylamine reagent were sequentially added. The mixture was vortexed, left to stand for 30 min, and the absorbance at 540 nm was measured to calculate nitrate reductase activity.

For the measurement of glutamine synthetase (GS) activity, 1 g of tobacco leaves was placed in a pre-chilled mortar, cut into small pieces, and ground on ice. The resulting homogenate was transferred to a centrifuge tube and centrifuged at 12,000 rpm for 20 min at 4 °C. A 0.7 mL aliquot of the supernatant was transferred to a new centrifuge tube and combined with 0.7 mL of ATP solution and 1.6 mL of reaction mixture. After thorough mixing, the reaction mixture was incubated at 37 °C for 30 min. Subsequently, 1 mL of chromogenic reagent was added, and the solution was vortexed to mix thoroughly, followed by centrifugation at 7300 rpm for 10 min. The supernatant was carefully collected, and its absorbance at 540 nm was measured.

Nicotine content was determined according to the method described in reference [23]. Dried tobacco leaf samples (0.1 g) were soaked in 1 mL of 10% (w/v) NaOH solution for 15 min and then extracted with an equal volume of dichloromethane, followed by vortexing. Nicotine content was subsequently quantified by LC-MS (Agilent 1260 Infinity II-7900; Agilent Technologies, Santa Clara, CA, USA). For each nitrogen application rate and topping treatment, data were obtained from three individual plants.

Fresh tobacco leaf samples were rapidly frozen in liquid nitrogen, ground into powder, and extracted in an ice bath with pre-chilled extraction buffer containing Tris-HCl. Then, the homogenate was centrifuged at 12,000× g for 15 min at 4 °C, and the supernatant was collected for use as the enzyme reaction system. Free ammonium concentration was determined by monitoring the glutamate dehydrogenase (GDH)-catalyzed reductive amination of α-ketoglutarate with ammonium ions, coupled to the oxidation of NADPH, which resulted in a decrease in absorbance at 340 nm. The rate of absorbance change at this wavelength was recorded, and the free ammonium concentration in the samples was quantified against an NH_4Cl standard curve. The results were expressed as $\mu g \cdot g^{-1}$ (FW)$\cdot h^{-1}$ [24]. The activities of sucrose phosphate synthase (SPS) and sucrose synthase (SUS) were measured using commercial assay kits (HRK1522 and HRK1520, respectively; Herui Biotechnology, Fuzhou, China). Each treatment was performed in triplicate, and mean values were calculated. All experimental procedures were conducted strictly in accordance with the manufacturer's instructions.

2.5. Metabolomic Profiling

At the budding stage, the largest middle leaves and corresponding roots (washed with distilled water to remove soil and root-associated debris) were collected from each nitrogen treatment before topping and at 10 days after topping. All samples were immediately frozen in liquid nitrogen and stored at −80 °C for subsequent transcriptomic and metabolomic analyses, with three biological replicates per treatment. Sample preparation and metabolomic data acquisition were performed by Shanghai Applied Protein Technology Co., Ltd. (Shanghai, China). Data were acquired and analyzed using ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) [25]. Metabolites were

identified and quantified using the MetWare Database (MWDB). Metabolomic data were analyzed as described by Wang et al. [26]. Differentially accumulated metabolites (DAMs) between comparison groups were screened based on fold change and Variable Importance in Projection (VIP) values derived from the OPLS-DA model, with screening criteria set at VIP ≥ 1 and $|\log_2(\text{fold change})| \geq 1$. Metabolomics-related results were visualized using the MetWare Cloud Platform (https://cloud.metware.cn).

2.6. Transcriptomic Profiling

Aliquots of the same samples used for metabolomic analysis were subjected to transcriptomic analysis. Total RNA extraction, library construction, and sequencing were performed by Shanghai Applied Protein Technology Co., Ltd. (Shanghai, China). After quality filtering, clean reads were aligned to the tobacco reference genome using HISAT2 (v2.2.1) [27] to generate BAM files. Gene expression levels were then quantified using featureCounts, and an expression matrix was generated. Transcripts per million (TPM) were used to estimate gene expression levels. The tobacco reference genome (*Nicotiana tabacum* cv. SR1, version 1) and corresponding annotation files were downloaded from the Nicomics database (http://lifenglab.hzau.edu.cn/Nicomics/, accessed on 15 March 2025) [28]. Subsequently, functional annotation of the identified genes was performed using the eggNOG-mapper (v2.1.9) tool based on the eggNOG database [29]. Differential expression analysis was performed using the DESeq2 (v1.38.3) R package [30], with differentially expressed genes (DEGs) screened at $|\log_2(\text{fold change})| > 1$ and $p < 0.05$. Pearson correlation coefficients among samples within and between groups were calculated based on the TPM values of all genes across each sample, and sample correlation heatmaps and principal component analysis (PCA) were generated. Gene Ontology (GO) [31,32] enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) [33] pathway enrichment analysis were performed using the clusterProfiler (v4.17.0) R package [34].

2.7. Statistical Analysis and Data Integration

Statistical analyses were performed using IBM SPSS Statistics 26. Given the multi-factorial experimental design, two-way analysis of variance (ANOVA) was performed using the general linear model (GLM) univariate procedure to assess the main effects of leaf position, topping (or growth stage), and nitrogen application rate, as well as their interactions (leaf position × nitrogen application rate, topping × nitrogen application rate, and stage × nitrogen application rate). When the interaction was significant, simple effects analysis was performed to examine the effect of one factor at each level of the other factor, followed by Duncan's post hoc test for multiple comparisons. When the interaction was not significant, the main effects were interpreted directly, and comparisons among levels of each factor were further performed using one-way ANOVA with Duncan's post hoc test after fixing the levels of the other factor via the "Select Cases" function in SPSS. A significance level of $p < 0.05$ was applied. Chemical structures of metabolites were visualized using ChemDraw (v20.0), and figures were assembled and refined using Adobe Illustrator (v26.4.1).

3. Results

3.1. Effects of Different Nitrogen Application Rates and Topping Treatments on Key Quality Traits and Physiological Characteristics of Tobacco

3.1.1. Key Agronomic Traits of Tobacco at Different Growth Stages Under Different Nitrogen Application Rates

As shown in Figure 1, all measured agronomic traits increased with increasing nitrogen application rate. Significant differences in plant height, stem girth, effective leaf number, and

internode length were detected among nitrogen treatments at the rosette, vigorous growing, and budding stages (Figure 1I–XII). For fifth-leaf length and width, significant differences among nitrogen treatments were observed at the rosette and vigorous growing stages. At the budding stage, no significant differences in fifth-leaf length or width were found between the medium- and high-nitrogen treatments, whereas both differed significantly from the low-nitrogen treatment (Figure 1XV,XVIII). These results indicate that nitrogen application exerts a stage-dependent regulatory effect on tobacco growth. At the rosette and vigorous growing stages, agronomic traits were highly responsive to nitrogen supply. After the onset of the budding stage, plant height, stem girth, effective leaf number, and internode length continued to respond to nitrogen application rate; however, fifth-leaf length and width no longer differed significantly between the medium- and high-nitrogen treatments. Therefore, ensuring adequate nitrogen supply before the budding stage is critical for achieving high-yielding tobacco plants, whereas nitrogen application can be appropriately reduced after the budding stage to improve nitrogen use efficiency.

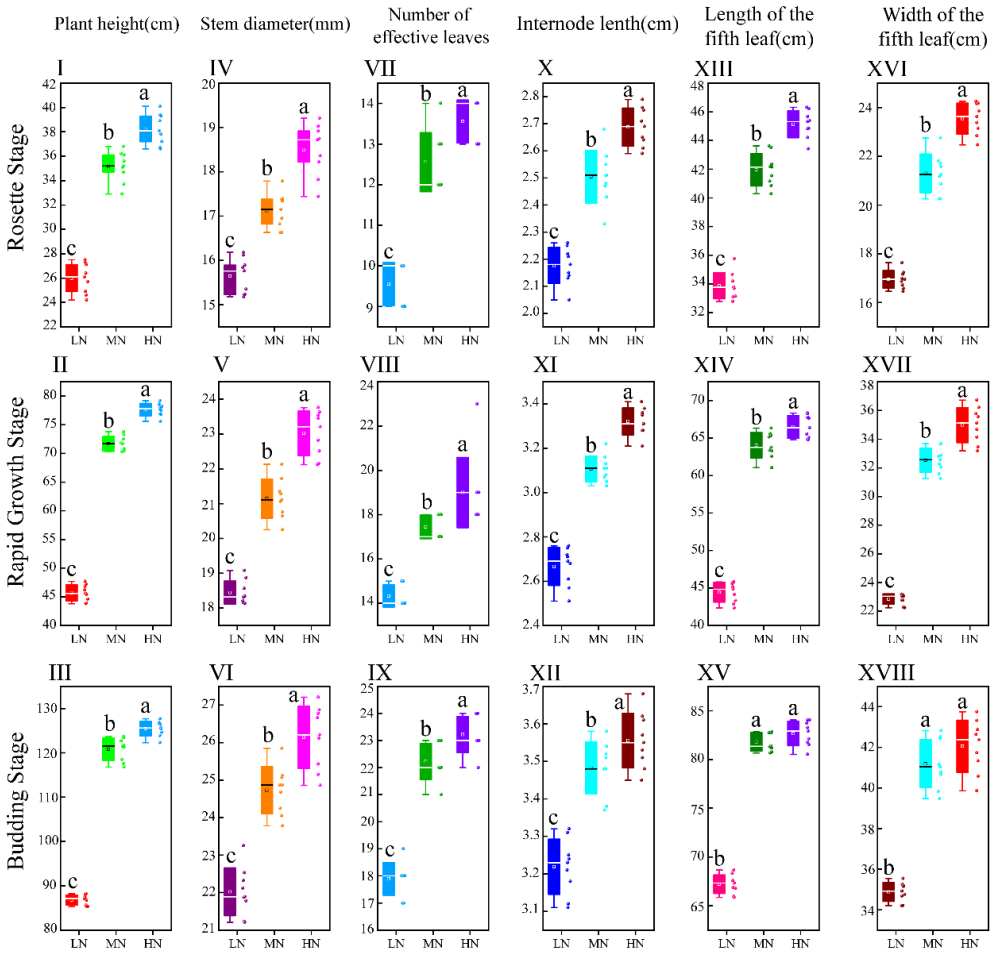


Figure 1. Major agronomic traits of tobacco at different growth stages under different nitrogen concentrations. (**I–III**) Plant height. (**IV–VI**) Stem diameter. (**VII–IX**) Number of effective leaves. (**X–XII**) Internode length. (**XIII–XV**) Length of the fifth leaf (cm). (**XVI–XVIII**) Width of the fifth leaf (cm). LN, low nitrogen; MN, medium nitrogen; HN, high nitrogen. Different lowercase letters above the boxplots indicate significant differences ($p < 0.05$) among nitrogen treatments at the same growth stage based on Duncan's multiple range test. Each column represents the measured values of the same agronomic trait at different growth stages under different nitrogen treatments.

3.1.2. Effects of Nitrogen Supply on Quality of Mature Leaves from Different Positions

Two-way ANOVA showed that leaf position significantly affected total nitrogen, soluble sugar, potassium, and chloride contents, but had no significant effect on total alkaloid or total sugar contents. Nitrogen application rate significantly affected total nitrogen, total alkaloid, total sugar, soluble sugar, potassium, and chloride contents. The leaf position × nitrogen application rate interaction significantly affected potassium contents, but had no significant effect on total alkaloid, total sugar, total nitrogen, soluble sugar, or chloride contents (Table S1).

Nitrogen application significantly affected total nitrogen content and total alkaloid content in tobacco leaves. Within the same leaf position, both total nitrogen content and total alkaloid content increased with increasing nitrogen application rate; however, total alkaloid content did not differ significantly between the medium- and high-nitrogen treatments. At the same nitrogen level, total nitrogen content and total alkaloid content differed significantly among leaf positions. Under low-nitrogen conditions, total nitrogen content increased with ascending leaf position, with lower leaves containing significantly less nitrogen than upper leaves. Under medium-nitrogen conditions, total nitrogen content did not differ significantly between middle and lower leaves, but both were significantly lower than that of upper leaves, possibly due to higher metabolic activity and greater nitrogen demand in upper leaves than in lower and middle leaves. Under high-nitrogen conditions, however, no significant differences in total nitrogen content were observed among the three leaf positions (Figure 2I). Under low-nitrogen conditions, total alkaloid content tended to decrease with ascending leaf position, although the differences were not statistically significant. Under medium-nitrogen conditions, total alkaloid content in middle leaves was significantly lower than in both upper and lower leaves. Under high-nitrogen conditions, although middle leaves exhibited the highest total alkaloid content, no significant differences were found among leaf positions (Figure 2II).

Except for the total sugar content of the lower leaves and the soluble sugar content of the upper leaves, the total sugar content of each position (Figure 2III) and the soluble sugar content (Figure 2IV) significantly decreased with increasing nitrogen concentration, possibly because the increase in nitrogen content enhanced related secondary metabolic activities, thereby consuming energy and carbon skeletons. Under the same nitrogen concentration, there was no significant difference in total sugar content among leaves at different positions, and under low and medium nitrogen conditions, there was no significant difference in soluble sugar content among leaves at different positions; under high nitrogen conditions, the middle leaves were significantly lower than the lower leaves. Overall, with the increase in nitrogen application rate, the accumulation of carbohydrates exhibited an inhibitory effect.

Within the same leaf position, potassium content decreased significantly with increasing nitrogen application. Potassium participates in nitrogen assimilation and enzyme activation and competes with NH_4^+ for uptake, which may explain the reduction in potassium content under higher nitrogen supply. At the same nitrogen level, potassium content in lower leaves was significantly higher than that in upper and middle leaves (Figure 2V), indicating that nitrogen application should be maintained at an appropriate level, because excessive nitrogen fertilization reduces potassium content and consequently impairs leaf combustibility. Under low- and medium-nitrogen conditions, chloride content in upper and middle leaves was significantly lower than that in lower leaves, with the lowest values observed under the medium-nitrogen treatment. In lower leaves, chloride content increased with increasing nitrogen level, although the differences were not statistically significant. Under high-nitrogen conditions, chloride content in middle leaves was significantly lower than

that in lower leaves (Figure 2VI). These results suggest that moderate nitrogen application helps coordinate chloride content in tobacco leaves, thereby improving leaf combustibility.

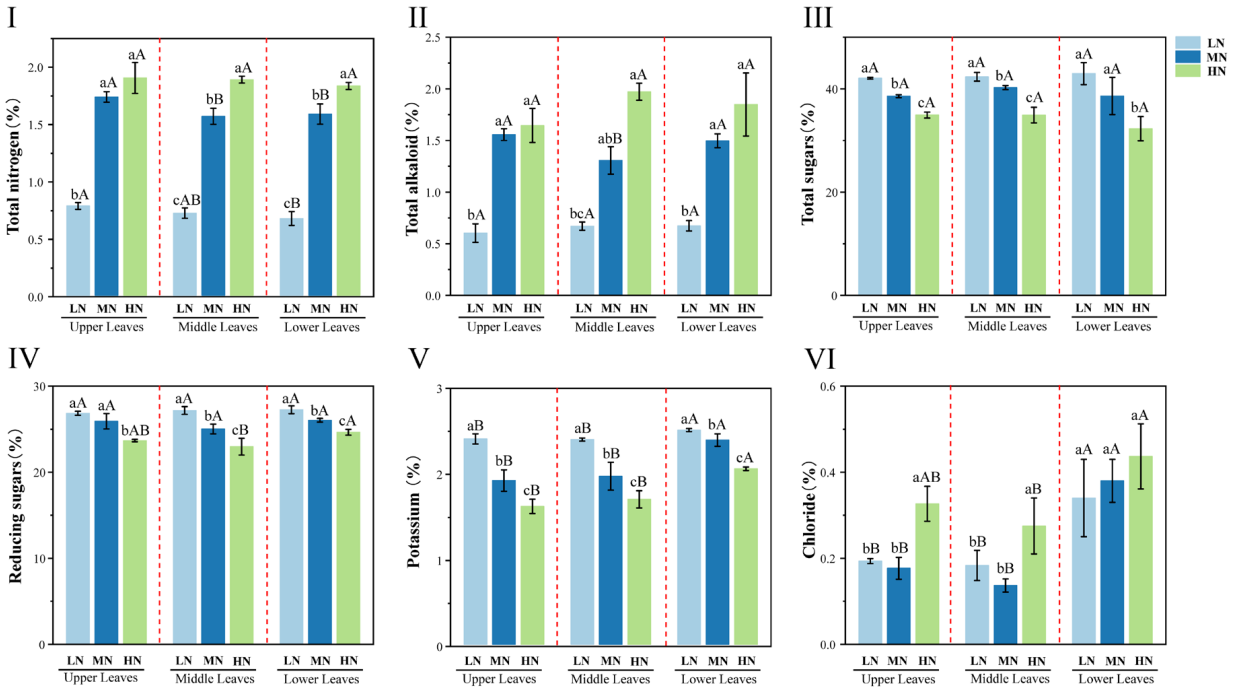


Figure 2. Bar charts showing the effects of nitrogen supply on the quality of mature leaves from different positions. (**I**) Total nitrogen content (%). (**II**) Total alkaloid content (%). (**III**) Total sugar content (%). (**IV**) Reducing sugar content (%). (**V**) Potassium content (%). (**VI**) Chloride content (%). LN, low nitrogen; MN, medium nitrogen; HN, high nitrogen. Data are presented as the mean ± standard deviation (SD). Different lowercase letters above the grouped bars indicate significant differences in each quality indicator among nitrogen treatments within the same leaf position, and different uppercase letters indicate significant differences among leaf positions within the same nitrogen treatment, based on Duncan's multiple range test ($p < 0.05$).

3.1.3. Effects of Topping Treatment on Carbon and Nitrogen Metabolism in Tobacco

Two-way ANOVA showed that both topping and nitrogen application rate significantly affected nicotine content, free ammonium content, nitrate reductase activity (NR), and glutamine synthetase activity (GS), whereas the topping × nitrogen application rate interaction significantly affected nicotine content, nitrate reductase activity, and glutamine synthetase activity, but had no significant effect on free ammonium content (Table S2).

Before topping, nicotine content and nitrate reductase activity increased with increasing nitrogen application, with significant differences among all treatments (Figure 3I,III). Glutamine synthetase activity also increased with rising nitrogen supply and differed significantly among treatments before topping; however, no significant difference was detected between the medium- and high-nitrogen treatments after topping (Figure 3IV). Compared with the pre-topping stage, nicotine content, NR activity, and GS activity all increased significantly after topping across all nitrogen treatments. Free ammonium content under the medium-nitrogen treatment was significantly lower than that under both the high- and low-nitrogen treatments before and after topping, and topping significantly reduced free ammonium content across all nitrogen treatments (Figure 3II).

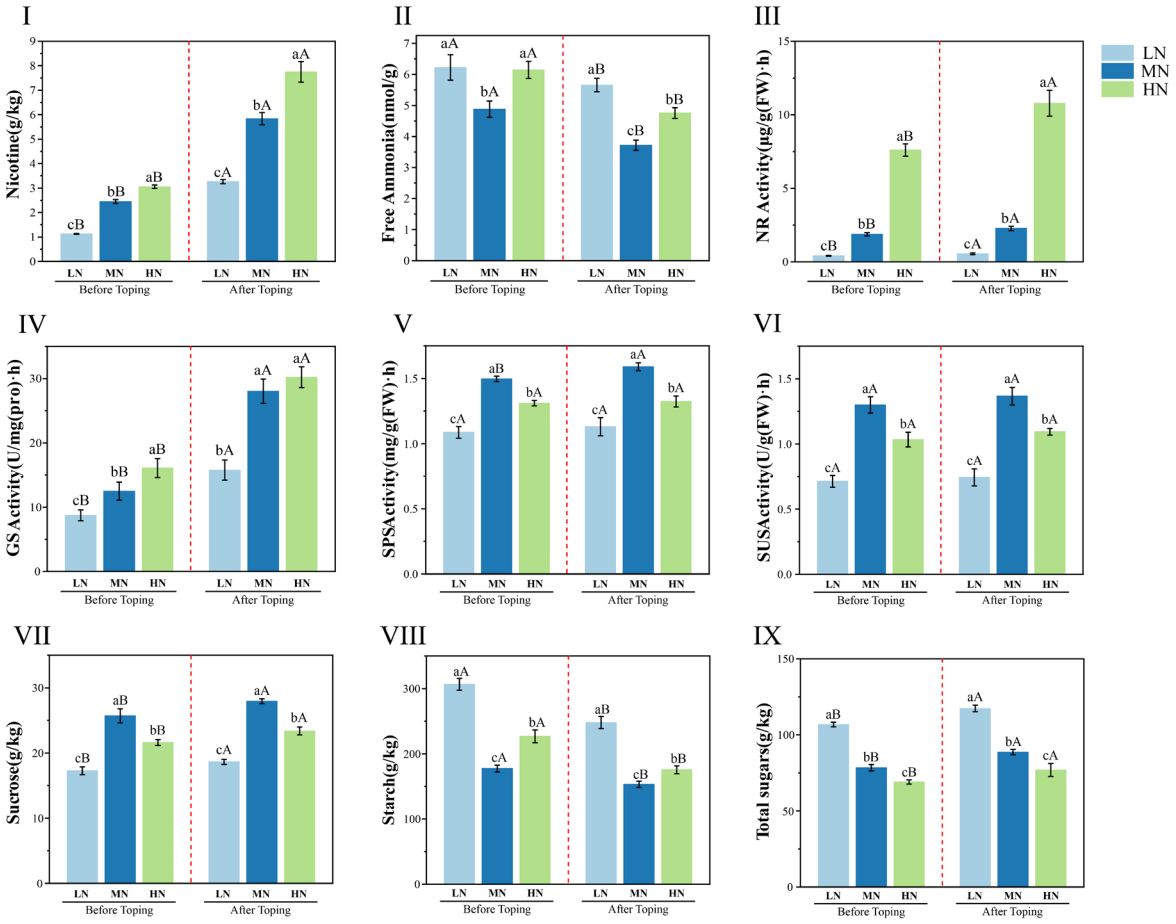


Figure 3. Effects of topping treatment on carbon and nitrogen metabolism in tobacco. (**I**) Nicotine content (g/kg). (**II**) Free ammonia content (nmol/g). (**III**) Nitrate reductase activity (μg/g(FW)·h). (**IV**) Glutamine synthetase activity (U/mg(pro)·h). (**V**) Sucrose phosphate synthase activity (mg/g(FW)·h). (**VI**) Sucrose synthase activity (U/g(FW)·h). (**VII**) Sucrose content (g/kg). (**VIII**) Starch content (g/kg). (**IX**) Total sugar content (g/kg). LN, low nitrogen; MN, medium nitrogen; HN, high nitrogen. Data are presented as the mean ± standard deviation (SD). Different lowercase letters above the bars indicate significant differences among nitrogen treatments (LN, MN, and HN) within the same topping stage (before or after topping), and different uppercase letters indicate significant differences between before and after topping within the same nitrogen treatment, based on Duncan's multiple range test ($p < 0.05$).

Two-way ANOVA showed that topping significantly affected sucrose phosphate synthase (SPS) activity, sucrose content, starch content, and total sugar content, but had no significant effect on sucrose synthase (SUS) activity. Nitrogen application rate significantly affected all five indicators. The topping × nitrogen application rate interaction significantly affected starch content and total sugar content, but had no significant effect on SPS activity, SUS activity, or sucrose content (Table S3).

Before and after topping, sucrose phosphate synthase and sucrose synthase activities differed significantly among nitrogen treatments, with the highest enzyme activities and sucrose content observed under the medium-nitrogen treatment. However, a significant increase in SPS activity after topping relative to before topping was observed only under the medium-nitrogen condition (Figure 3V,VI). Sucrose content was highest under the medium-nitrogen treatment both before and after topping, and topping significantly increased sucrose content under all nitrogen treatments, whereas starch content showed the opposite trend (Figure 3VII,VIII). Total sugar content decreased with increasing nitrogen application but increased significantly after topping (Figure 3IX). Collectively, these results indicate

that soluble sugar and starch contents can be modulated by adjusting nitrogen application rate and topping treatment, thereby improving tobacco leaf quality.

3.1.4. Effects of Different Nitrogen Application Rates on Rhizosphere Soil Physicochemical Properties at Different Growth Stages

Two-way ANOVA showed that nitrogen application rate had no significant effect on soil pH but significantly affected soil organic matter (SOM), total nitrogen, alkali-hydrolyzable nitrogen, available phosphorus, available potassium, and water-soluble chloride contents, whereas stage significantly affected all of the above indicators. The stage × nitrogen application rate interaction significantly affected soil pH, organic matter, available phosphorus, available potassium, and water-soluble chloride contents but had no significant effect on total nitrogen or alkali-hydrolyzable nitrogen contents (Table S4).

At the rosette and vigorous growing stages, rhizosphere soil pH increased with increasing nitrogen application, with no significant difference between medium- and high-nitrogen treatments, whereas both differed significantly from the low-nitrogen treatment. At the budding stage, however, pH significantly decreased with increasing nitrogen application (Figure S1I). Soil organic matter content under medium-nitrogen treatment was significantly higher than that under both low- and high-nitrogen treatments across all growth stages, with the lowest values observed under low-nitrogen treatment. SOM content exhibited a decreasing then increasing trend over the three growth stages; under low-nitrogen conditions, SOM at the vigorous growing stage was significantly lower than that at the rosette and budding stages, whereas under medium- and high-nitrogen conditions, SOM at the vigorous growing stage was significantly lower than that at the rosette stage, with no significant change between the rosette and budding stages (Figure S1II). Total nitrogen (g/kg) and hydrolyzable nitrogen (mg/kg) contents in rhizosphere soil increased with increasing nitrogen application at the same growth stage. Except for total nitrogen content under low-nitrogen treatment, which differed significantly between the rosette and budding stages, no significant differences in total nitrogen or hydrolyzable nitrogen contents were observed across growth stages under the other treatments (Figure S1III,IV). Available phosphorus content showed no significant difference between low- and medium-nitrogen treatments except at the vigorous growing stage, whereas both differed significantly from the high-nitrogen treatment; significant differences in available phosphorus content among nitrogen treatments were observed at the vigorous growing stage (Figure S1V). At the same growth stage, soil available potassium content (mg/kg) was highest under medium-nitrogen treatment; however, no significant differences among nitrogen treatments were detected at the rosette stage, whereas significant differences were observed at the vigorous growing and budding stages. Under the same nitrogen concentration, available potassium content significantly increased with advancing growth stage, a trend consistent with that observed for potassium content in tobacco leaf quality (Figure S1VI). Except under low-nitrogen treatment, water-soluble chloride content exhibited a decreasing then increasing trend across growth stages, with the lowest values at the vigorous growing stage; under low-nitrogen treatment, water-soluble chloride content decreased with advancing growth stage, though the differences were not significant (Figure S1VII). At the rosette stage, nitrogen treatment exerted no significant effect on chloride content; at the vigorous growing stage, water-soluble chloride content was lowest under medium-nitrogen treatment; and at the budding stage, chloride content under high-nitrogen treatment was significantly higher than that under low- and medium-nitrogen treatments. Collectively, these results indicate that nitrogen treatment affects soil physicochemical properties, which in turn influence tobacco leaf quality, and that appropriate regulation of nitrogen application can modulate the contents of potassium, chloride, and other elements, thereby improving leaf quality.

3.1.5. Relationships Between Major Agronomic Traits and Mature Leaf Quality of Tobacco and Soil Physicochemical Properties Under Different Nitrogen Application Rates

To elucidate the relationships between soil physicochemical properties at different growth stages and the major agronomic traits and mature leaf quality of tobacco under varying nitrogen application rates, correlation analyses were performed. At the rosette stage, major agronomic traits—including stem diameter (Sd), plant height (Ph), number of effective leaves (Nel), fifth-leaf length (Ll), internode length (Il), and fifth-leaf width (Wl)—were positively correlated with soil organic matter (SOM), pH, total nitrogen (TN), and hydrolyzable nitrogen (AN) across nitrogen treatments (Figure 4A). Compared with the rosette stage, soil pH at the vigorous growing stage showed no correlation with agronomic traits, whereas available phosphorus (AP) was positively correlated with these traits (Figure 4B). At the budding stage, in contrast to the previous two stages, pH was negatively correlated with agronomic traits, while water-soluble chloride (WSC) was positively correlated with them (Figure 4C). Collectively, these results suggest that appropriate application of nitrogen and phosphorus fertilizers helps improve tobacco agronomic traits and yield.

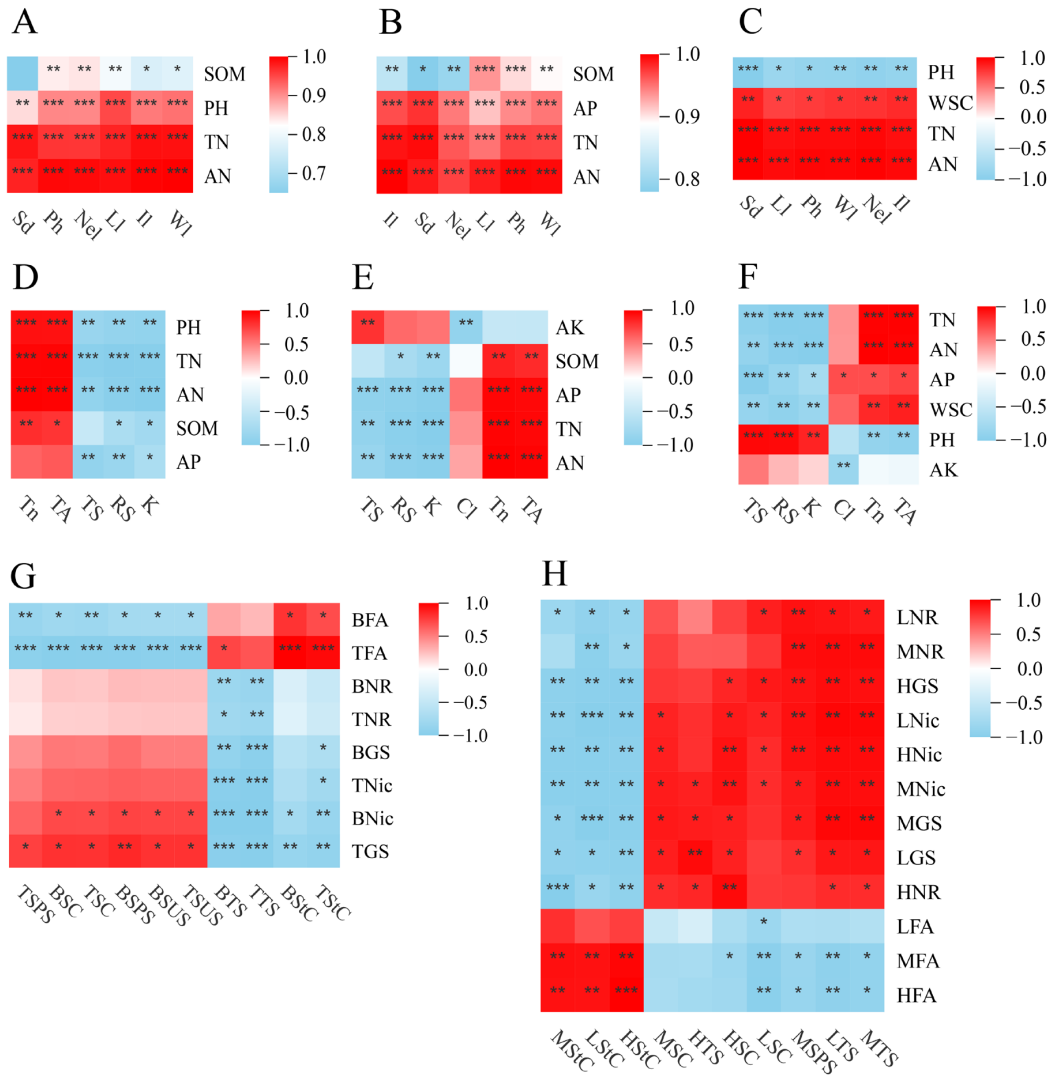


Figure 4. Correlation analysis of soil physicochemical properties at different growth stages with major agronomic traits at corresponding growth stages (**A**–**C**), correlation analysis of soil physicochemical properties at different growth stages with mature tobacco leaf quality under different nitrogen application rates (**D**–**F**), and correlation analysis of carbon and nitrogen metabolism under topping

treatment and different nitrogen application rates (**G**,**H**). SOM, soil organic matter content; pH, soil pH; TN, soil total nitrogen content; AN, hydrolyzable nitrogen content; AP, soil available phosphorus content; WSC, soil water-soluble chloride content; AK, soil available potassium content; Ph, plant height; Sd, stem diameter; Nel, number of effective leaves; Ll, fifth-leaf length; Il, internode length; Tn, total nitrogen content of mature leaves; TA, total alkaloid content of mature leaves; TS, total sugar content of mature leaves; RS, reducing sugar content of mature leaves; Cl, chloride content of mature leaves; K, potassium content of mature leaves. In the carbon and nitrogen metabolism correlation analysis, L, low-nitrogen treatment. M, medium-nitrogen treatment; H, high-nitrogen treatment; B, before topping; T, after topping; FA, free ammonia content; NR, nitrate reductase activity; GS, glutamine synthetase activity; Nic, nicotine content; SPS, sucrose phosphate synthase activity. SUS, sucrose synthase activity. SC, sucrose content; TS, total sugar content; StC, starch content. Asterisks indicate significant differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

With increasing nitrogen application, leaf potassium (K), reducing sugar (RS), and total sugar (TS) contents were negatively correlated with soil pH, total nitrogen (TN), hydrolyzable nitrogen (AN), soil organic matter (SOM), and available phosphorus (AP) at the rosette stage, whereas total alkaloid (TA) and leaf total nitrogen (Tn) contents showed the opposite trend (Figure 4D). Compared with the rosette stage, soil pH at the vigorous growing stage exhibited no correlation with leaf quality traits; however, available potassium (AK) was positively correlated with leaf potassium (K), reducing sugar (RS), and total sugar (TS) contents, and negatively correlated with total alkaloid (TA), leaf total nitrogen (Tn), and chloride (Cl) (Figure 4E). At the budding stage, soil total nitrogen, hydrolyzable nitrogen, available phosphorus, and water-soluble chloride were negatively correlated with leaf potassium, reducing sugar, and sucrose contents, and positively correlated with leaf chloride (Cl), leaf total nitrogen (Tn), and total alkaloid (TA) contents, whereas pH and available potassium showed the opposite correlations (Figure 4F).

3.1.6. Relationships Between Carbon and Nitrogen Metabolism in Tobacco Leaves Under Different Nitrogen Application Rates and Topping Treatments

To elucidate the relationships between carbon and nitrogen metabolism in tobacco leaves under different nitrogen application rates and topping treatments, correlation analyses were conducted among physiological indicators related to nitrogen and carbon metabolism across the various treatments. As nitrogen application increased, free ammonia content (FA) both before and after topping was positively correlated with total sugar content (TS) and starch content (StC) but negatively correlated with sucrose phosphate synthase (SPS) activity, sucrose content (SC), and sucrose synthase (SUS) activity. In contrast, nitrate reductase (NR) activity, glutamine synthetase (GS) activity, and nicotine content (Nic) were correlated with the aforementioned leaf quality traits in a manner opposite to that of free ammonia (Figure 4G). Topping significantly affected carbon and nitrogen metabolism across nitrogen application rates. After topping, free ammonia content was positively correlated with starch content and was lower than before topping, whereas it was negatively correlated with total sugar and sucrose contents. The correlations of NR activity, GS activity, and nicotine content were opposite to those of free ammonia. Notably, SPS activity was correlated with nitrogen metabolism only under the medium-nitrogen treatment (Figure 4H), consistent with the highest sucrose content observed under this condition (Figure 3VII). Collectively, these results indicate that topping enhanced both carbon and nitrogen metabolism. Carbon metabolism primarily consumed starch and increased the contents of soluble sugars such as sucrose, whereas nitrogen metabolism mainly utilized free ammonia to promote nicotine accumulation and enhanced nitrogen assimilation to increase total nitrogen content, thereby improving tobacco leaf quality.

3.2. Effects of Different Nitrogen Application Rates and Topping Treatments on Gene Expression in Tobacco Roots and Leaves

3.2.1. Transcriptomic Data and Distribution of Differentially Expressed Genes

To better understand the regulatory mechanisms by which different nitrogen application rates and topping treatments affect gene expression in tobacco, RNA-Seq was performed on leaves and roots before topping, as well as on leaves and roots after topping, under low-, medium-, and high-nitrogen treatments. The Q30 base quality (percentage of bases with a sequencing error rate ≤ 0.1%) exceeded 95.59% for all RNA-Seq libraries, including the supplementary sequencing data (Table S5). In principal component analysis (PCA), the first principal component (PC1) accounted for 31.59% of the total variance, and the second principal component (PC2) accounted for 10.40%, with clear separation among samples under different nitrogen application rates and topping treatments (Figure 5A). Correlation analysis among samples revealed high correlations between replicates for all samples except NLT24LT1 and NMT24RT1, indicating that the data were reliable and suitable for further analysis (Figure 5B).

To characterize transcriptomic differences in tobacco roots and leaves under different nitrogen application rates and between pre- and post-topping stages, 14 pairwise comparisons were established. In the NLB24L_vs_NMB24L comparison, 11,128 DEGs were identified (4912 upregulated and 6216 downregulated); in NMB24L_vs_NHB24L, 3414 DEGs (2707 upregulated and 707 downregulated); in NLT24L_vs_NMT24L, 12,723 DEGs (6549 upregulated and 6174 downregulated); in NMT24L_vs_NHT24L, 5091 DEGs (2507 upregulated and 2584 downregulated); in NLB24R_vs_NMB24R, 1822 DEGs (908 upregulated and 914 downregulated); in NMB24R_vs_NHB24R, 49 DEGs (27 upregulated and 22 downregulated); in NLT24R_vs_NMT24R, 12,090 DEGs (5711 upregulated and 6379 downregulated); in NMT24R_vs_NHT24R, 17 DEGs (6 upregulated and 11 downregulated); in NLB24L_vs_NLT24L, 13,390 DEGs (6580 upregulated and 6810 downregulated); in NMB24L_vs_NMT24L, 10,861 DEGs (6285 upregulated and 4576 downregulated); in NHB24L_vs_NHT24L, 9583 DEGs (4041 upregulated and 5542 downregulated); in NLB24R_vs_NLT24R, 11,156 DEGs (5875 upregulated and 5281 downregulated); in NMB24R_vs_NMT24R, 1277 DEGs (797 upregulated and 480 downregulated); and in NHB24R_vs_NHT24R, 2357 DEGs (1603 upregulated and 754 downregulated). With increasing nitrogen application, the number of DEGs in the low-nitrogen versus medium-nitrogen comparisons was markedly higher than that in the medium-nitrogen versus high-nitrogen comparisons. At the same nitrogen concentration, topping resulted in more DEGs in leaves than in roots, indicating that gene expression in tobacco is more sensitive to the transition from low to medium nitrogen and that topping exerts a stronger effect on gene expression in leaves than in roots (Figure 5C). To further investigate the effects of increasing nitrogen application before and after topping, as well as the effects of topping at the same nitrogen concentration, on gene expression in tobacco roots and leaves, union sets of DEGs from the 14 comparisons were generated. NBLU denotes the union set of DEGs in leaves in response to increasing nitrogen application before topping; NBRU denotes the union set in roots in response to increasing nitrogen application before topping; NTLU denotes the union set in leaves in response to increasing nitrogen application after topping; NTRU denotes the union set in roots in response to increasing nitrogen application after topping; TLU denotes the union set of DEGs in leaves after topping at the same nitrogen concentration; and TRU denotes the union set in roots after topping at the same nitrogen concentration.

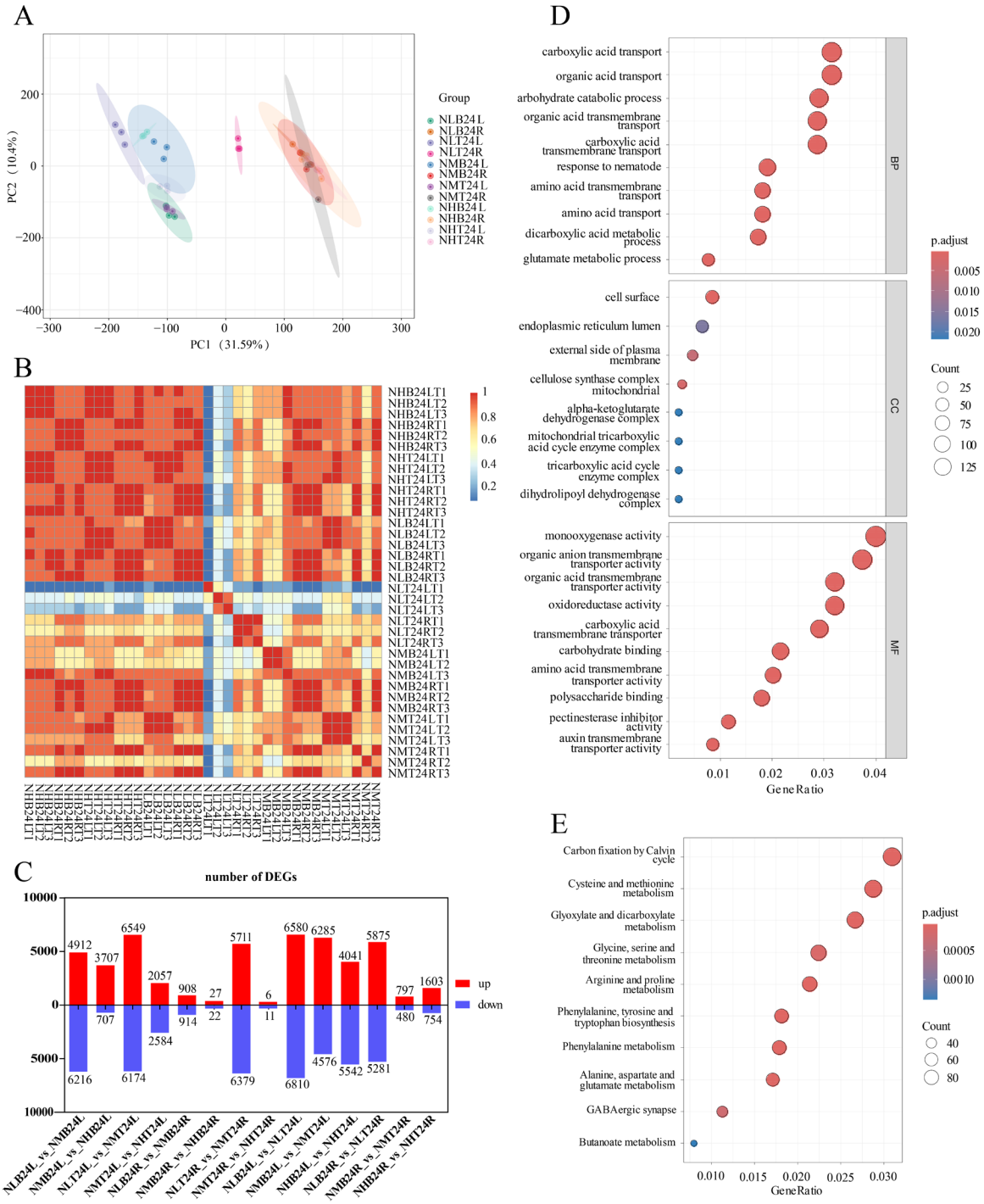


Figure 5. Effects of different nitrogen application rates and topping treatments on the transcriptomes of tobacco roots and leaves. (**A**) Principal component analysis (PCA) of transcriptomes across treatments. (**B**) Correlation analysis among samples. (**C**) Numbers of up-regulated and down-regulated genes in each comparison group. (**D**) GO enrichment analysis of the TLU differentially expressed gene union set. (**E**) KEGG enrichment analysis of the TLU differentially expressed gene union set.

3.2.2. Transcriptional Differences in Roots and Leaves in Response to Increasing Nitrogen Application Before and After Topping

To investigate the effects of increasing nitrogen application on tobacco roots and leaves before and after topping, the union sets of differentially expressed genes (DEGs) NBLU, NBRU, NTLU, and NTRU were subjected to GO and KEGG enrichment analyses. GO

enrichment analysis revealed that DEGs in roots and leaves before and after topping were mainly enriched in the Biological Process (BP) and Molecular Function (MF) categories.

In the NBLU set, the top 10 significantly enriched BP terms included response to nitrate (GO:0010167), auxin-activated signaling pathway (GO:0009734), and others; the top 10 significantly enriched MF terms included calcium ion binding (GO:0005509), carbohydrate binding (GO:0030246), auxin transmembrane transporter activity (GO:0080161), and others (Figure S2A; Table S6). In the NTLU set, the top 10 significantly enriched BP terms included organic acid transport (GO:0015849), amino acid transport (GO:0006865), and others; the top 10 significantly enriched MF terms included organic acid transmembrane transporter activity (GO:0005342), amino acid transmembrane transporter activity (GO:0015171), auxin binding (GO:0010011), and others (Figure S2B; Table S8). KEGG pathway analysis revealed that DEGs in the NBLU set before topping were mainly enriched in amino acid metabolism pathways (Figure S2C), whereas those in the NTLU set were mainly enriched in amino acid metabolism and secondary metabolite metabolism pathways (Figure S2D; Tables S12 and S14). Collectively, these results indicate that before topping, increasing nitrogen concentration accelerated nitrate metabolism and carbohydrate metabolism in tobacco leaves, whereas after topping, increasing nitrogen concentration accelerated amino acid metabolism and secondary metabolite metabolism.

In the NBRU set, the top 10 significantly enriched BP terms included hormone catabolic process (GO:0042447), gibberellin catabolic process (GO:0045487), and others; the top MF terms included polysaccharide binding (GO:0030247) and others (Figure S3A; Table S7). In the NTRU set, the top 10 significantly enriched BP terms included response to iron ion (GO:0010039), response to herbivore (GO:0080027), lignin metabolic process (GO:0009808), and secondary metabolite metabolism; MF terms were mainly enriched in oxidoreductase activity (GO:0016722), carbohydrate binding (GO:0030246), and others (Figure S4A; Table S9). KEGG enrichment analysis revealed that genes in the NBRU set were mainly enriched in pathways such as pentose and glucuronate interconversions (Figure S3C; Table S13), whereas genes in the NTRU set were mainly enriched in amino acid metabolism, carbon metabolism, and secondary metabolite metabolism pathways (Figure S4B; Table S15). These results indicate that before topping, tobacco roots responded to increasing nitrogen concentration primarily through hormone-related processes, whereas after topping, roots responded to increasing nitrogen concentration through amino acid metabolism and secondary metabolite metabolism.

3.2.3. Effects of Topping Treatment on the Transcriptomes of Tobacco Roots and Leaves

To investigate the effects of topping treatment on tobacco roots and leaves, enrichment analyses were performed on the union sets of differentially expressed genes (DEGs) TLU and TRU. GO enrichment analysis revealed that the DEGs were mainly enriched in the Biological Process (BP) and Molecular Function (MF) categories.

In the TLU set, the top 10 significantly enriched BP and MF terms primarily included carboxylic acid transport, amino acid transport, organic acid transport, and carbohydrate metabolism, such as carboxylic acid transport (GO:0046942), organic acid transport (GO:0015849), amino acid transmembrane transport (GO:0003333), carbohydrate catabolic process (GO:0016052), organic acid transmembrane transporter activity (GO:0005342), carbohydrate binding (GO:0030246), and amino acid transmembrane transporter activity (GO:0015171) (Figure 5D; Table S10). In the TRU set, the top 10 significantly enriched BP and MF terms mainly included response to metal ions, carbohydrate transport, and amino acid metabolism, such as response to iron ion (GO:0010039), carbohydrate transport (GO:0008643), glutathione metabolic process (GO:0006749), and carbohydrate transmembrane transporter activity (GO:0015144) (Figure S3B; Table S11). KEGG enrichment analysis

revealed that the top 10 significantly enriched pathways for genes in both TLU and TRU were mainly associated with amino acid metabolism and the Calvin cycle, including cysteine and methionine metabolism, alanine, aspartate and glutamate metabolism, and carbon fixation in the Calvin cycle. These results indicate that topping enhanced carbon metabolism and amino acid metabolism in both roots and leaves of tobacco (Figures 5E and S3D; Tables S16 and S17).

3.3. Effects of Different Nitrogen Application Rates and Topping Treatments on the Metabolomes of Tobacco Roots and Leaves

3.3.1. Metabolomic Data and Distribution of Differentially Accumulated Metabolites

To investigate the effects of different nitrogen application rates and topping treatments on the metabolomes of tobacco roots and leaves, metabolomic profiling was performed on root and leaf samples from the various treatment groups. OPLS-DA revealed clear separation among samples under different nitrogen application rates and topping treatments, with distinct clustering between root and leaf samples (Figure 6B). Permutation tests showed that all permuted points (red and blue) on the left were lower than the rightmost point, and the Y-axis intercept of the Q^2 regression line was below zero, indicating that the OPLS-DA models were reliable (Figure 6C). These results demonstrate that different nitrogen application rates and topping treatments exert profound effects on the metabolomes of tobacco roots and leaves, and that roots and leaves jointly respond to environmental changes through distinct metabolic pathways. A total of 1118 metabolites were detected under the mixed ion mode, which were classified into 17 categories: Alkaloids, Amino acids and Peptides, Carbohydrates, Coumarins, Diarylheptanoids, Fatty acids, Flavonoids, Isoflavonoids, Lignans, Phenanthrenoids, Phenolic acids (C6–C1), Phenylethanoids (C6–C2), Phenylpropanoids (C6–C3), Polyketides, Terpenoids, Xanthones, and others (Figure 6A; Table S18).

To investigate the effects of different nitrogen application rates and topping treatments on the metabolomes of roots and leaves, analysis of differentially accumulated metabolites (DAMs) was performed on samples from each nitrogen treatment and topping treatment group. A total of 766 DAMs were identified (Figure 6D; Table S19). The comparison groups for DAM analysis were consistent with those for differential gene expression analysis, and the same union set strategy used for differentially expressed genes was adopted for DAMs. The highest numbers of DAMs were observed in the low- versus medium-nitrogen comparisons and in the medium-nitrogen topping treatment comparisons, indicating that tobacco metabolism is most sensitive to medium-nitrogen conditions, particularly when medium-nitrogen treatment is combined with topping, which strongly perturbs the metabolomes of both roots and leaves.

3.3.2. Effects of Different Nitrogen Application Rates on Tobacco Root and Leaf Metabolism Before and After Topping

To elucidate the effects of different nitrogen application rates and topping treatments on tobacco metabolism, K-means clustering analysis was performed on the union sets of differentially accumulated metabolites (DAMs), and the numbers of upregulated and downregulated metabolites in response to increasing nitrogen concentration and topping were statistically analyzed. Before topping, as nitrogen concentration increased, the upregulated metabolites in tobacco leaves were mainly alkaloids, terpenoids, and fatty acid metabolites, while the downregulated metabolites also belonged predominantly to these three classes; however, the number of upregulated metabolites exceeded that of downregulated ones (Figure S5B). In roots before topping, as nitrogen concentration increased, most DAMs decreased in content, including terpenoids, fatty acids, alkaloids, amino acids, and carbohydrates, whereas the upregulated metabolites were mainly plant alkaloids, lignans, and

carbohydrates (Figure S5D). After topping, the most prominently altered DAMs in both roots and leaves were also terpenoids, alkaloids, and fatty acids; however, compared with before topping, the variety of DAM species increased in both roots and leaves (Figure S5F,H). These results indicate that with increasing nitrogen concentration, tobacco roots and leaves primarily adapt to nitrogen variation through differential regulation of distinct metabolite species within the same metabolite classes.

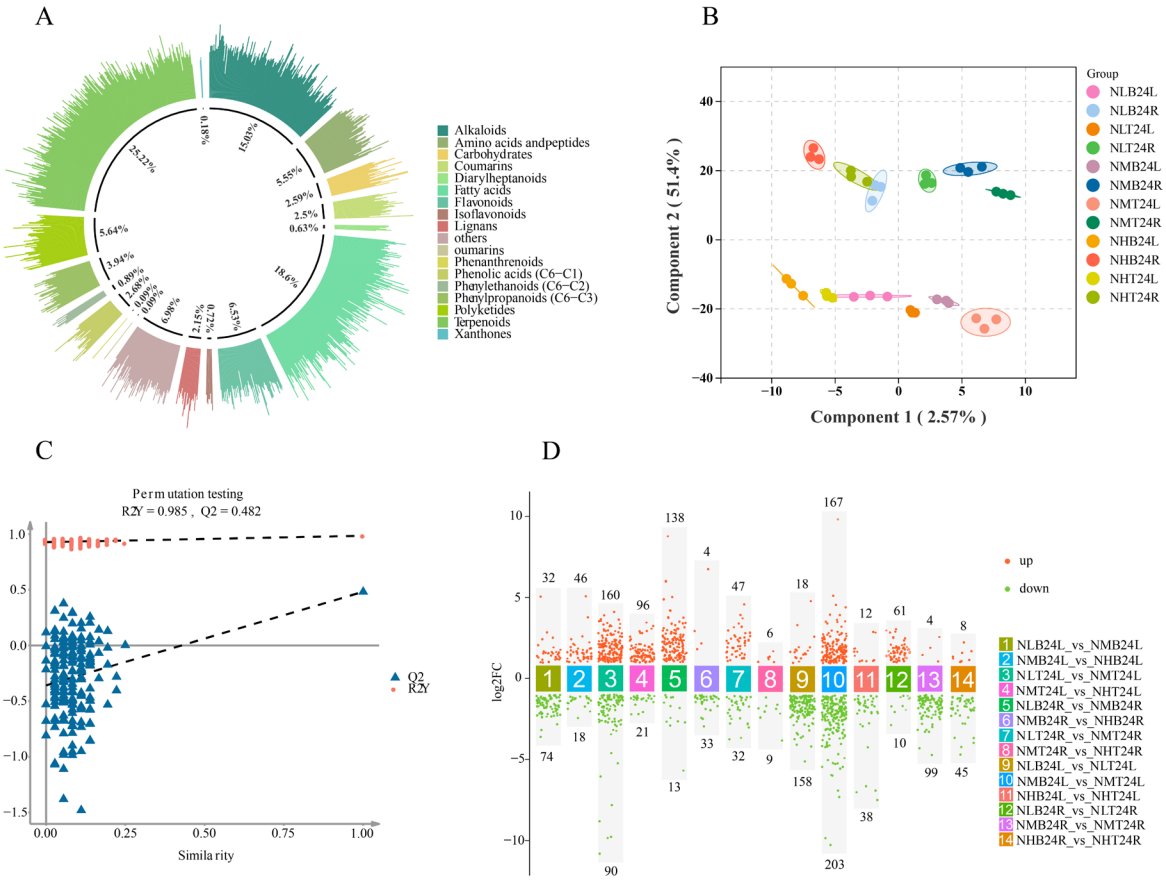


Figure 6. Effects of different nitrogen application rates and topping on the metabolomes of tobacco roots and leaves. (**A**) Proportion of metabolite classes. (**B**) OPLS-DA analysis of metabolomic data. (**C**) Permutation test of the OPLS-DA model. (**D**) Distribution of differentially accumulated metabolites in each comparison group.

3.3.3. Effects of Topping on Tobacco Root and Leaf Metabolism

Topping significantly altered metabolite accumulation patterns in tobacco, with the most prominent changes observed in terpenoids, alkaloids (including nicotine), and fatty acids. Overall, the diversity of differentially accumulated metabolites was greater in leaves than in roots (Figure S6B). Compared with nitrogen treatment alone, topping had a greater effect on the differential accumulation of terpenoids, alkaloids, lignans, amino acids, and other metabolites in leaves, and it also promoted alkaloid accumulation in roots (Figure S6D). Collectively, these results indicate that tobacco primarily responds to topping through the differential accumulation of specific metabolite species within classes such as alkaloids, amino acids, fatty acids, and terpenoids.

3.4. Weighted Gene Co-Expression Network Analysis (WGCNA)

To investigate potential regulatory relationships between metabolites and genes, WGCNA was first performed on the differentially accumulated metabolites using a soft-thresholding power of 6 (Figure S7A). Based on similar accumulation patterns, a total of

seven expression modules (excluding the grey module) were identified for the metabolites (Figure S7B). For genes, the top 75% of genes were retained based on median absolute deviation, followed by selection of the top 60% based on interquartile range (IQR). Using dynamic tree cutting and module merging algorithms, a total of 37 expression modules (excluding the grey module) were identified (Figure 7B). Representative metabolites were selected from each metabolite expression module for correlation analysis with gene module eigengenes. These metabolites included Nicotine, DL-Glutamine, D-Psicose, Jasmonic acid, 21-Hopen-3-one, h-gamma-Glu-Leu-OH, and Scoparone. Among these, the co-expression module most highly correlated with nicotine was MEdarkturquoise (Figure 7A). The dark-turquoise module contained 52 genes, with domain annotations predominantly comprising P450, Terpene_synth, and Terpene_synth_C (Table S20). To elucidate potential interactions among these genes, a gene interaction network was constructed for the 52 genes in the module (Figure 7C), in which nodes represent genes and edges represent connections between genes. Network analysis revealed that genes *Nta09g04330* and *Nta10g03250* exhibited the highest connectivity, and their Pfam annotations were Terpene_synth and Terpene_synth_C (Table S20).

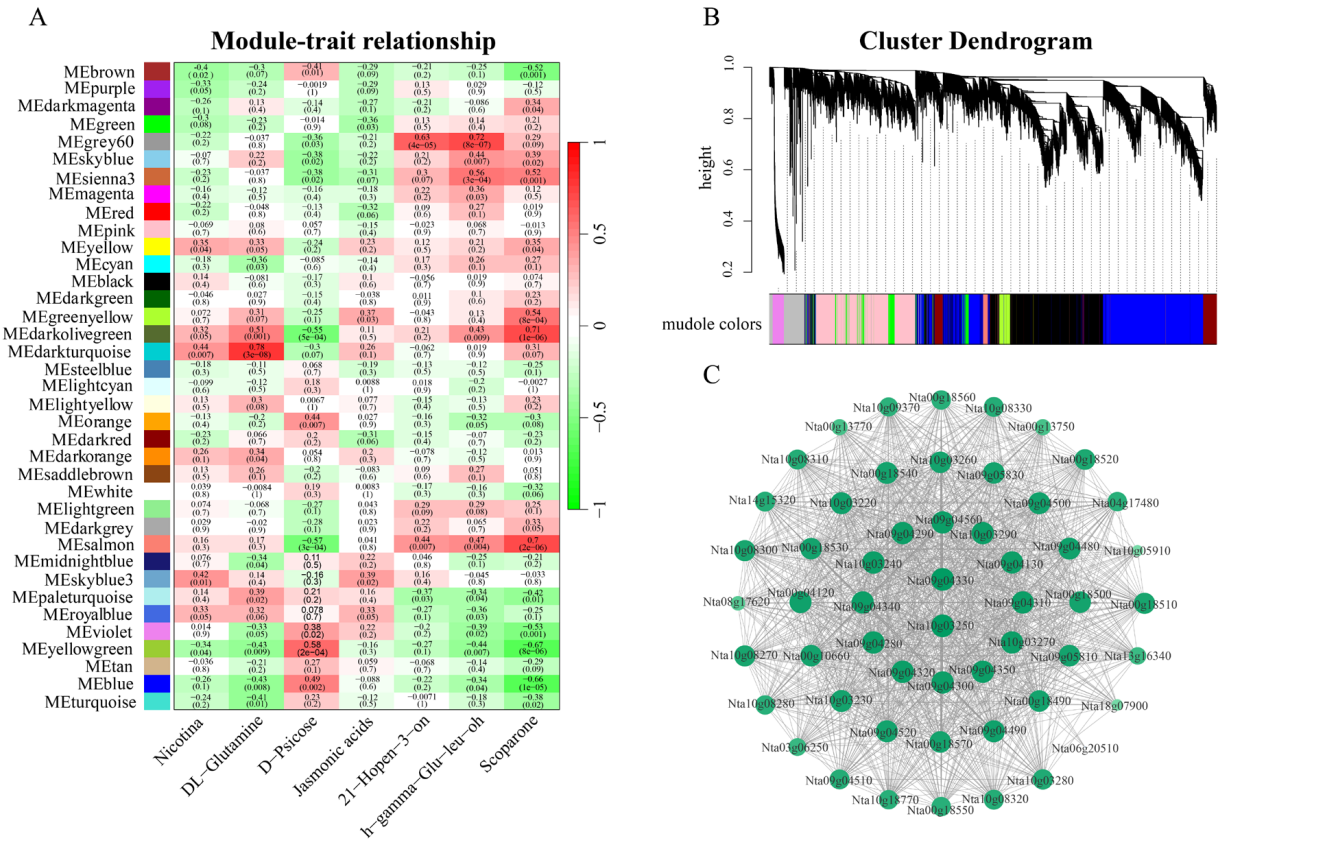


Figure 7. Weighted gene co-expression network analysis (WGCNA). (**A**) Correlation analysis between modules and metabolites. (**B**) Module clustering dendrogram of gene WGCNA analysis. (**C**) Gene co-expression network of the darkturquoise module.

3.5. Effects of Different Nitrogen Application Rates and Topping Treatments on Tobacco Physiology

3.5.1. Effects of Different Nitrogen Application Rates and Topping Treatments on Carbon and Nitrogen Metabolic Pathways in Tobacco Roots and Leaves

Different nitrogen application rates and topping treatments influenced carbon and nitrogen metabolism in tobacco roots and leaves, thereby affecting leaf quality. Gene expression levels were mapped to pathways related to carbon and nitrogen metabolism,

including starch degradation, sucrose degradation, sucrose synthesis, glucose metabolism, and nitrate assimilation (Figure 8).

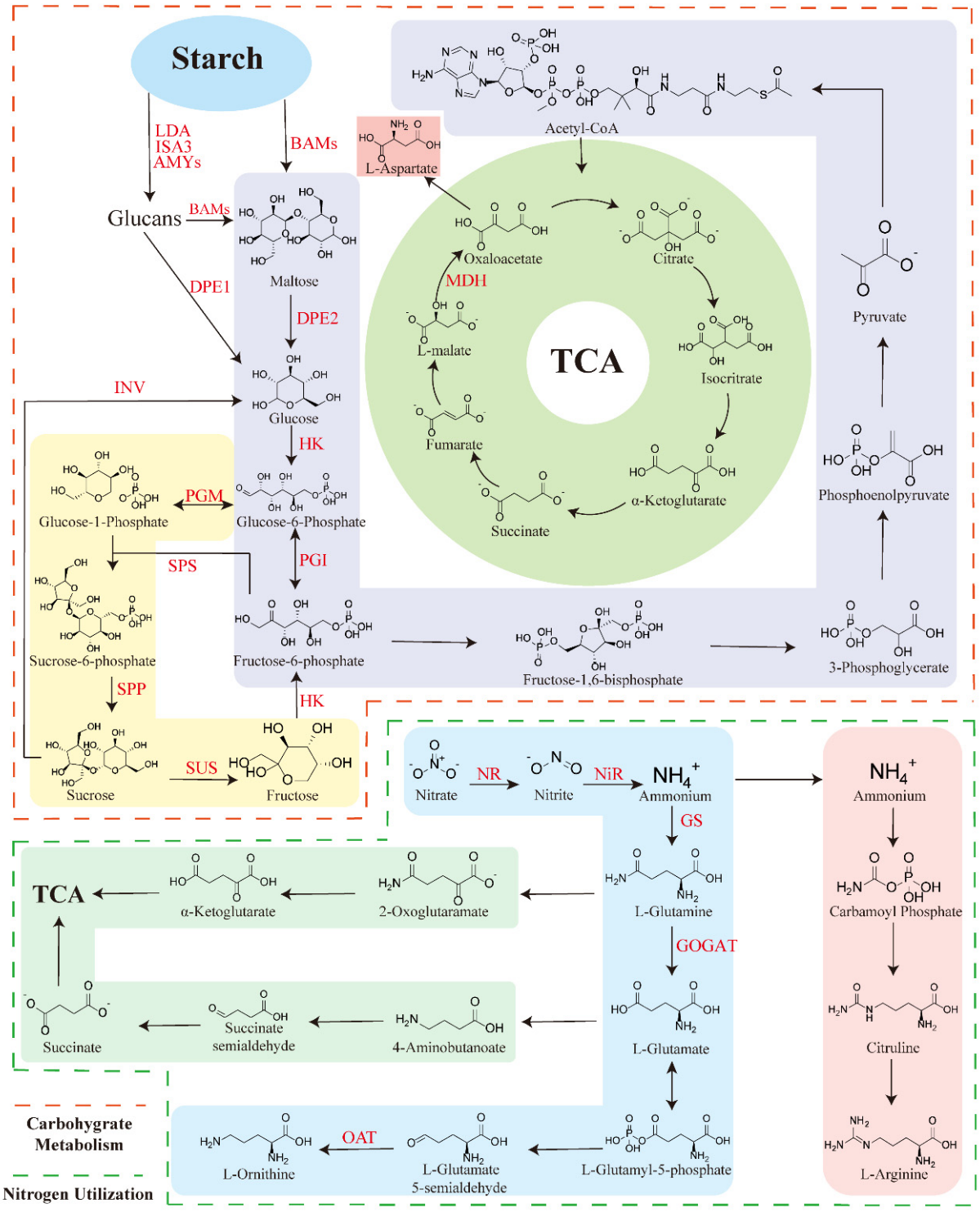


Figure 8. Effects of different nitrogen application rates and topping treatments on genes encoding enzymes involved in carbon and nitrogen metabolism in tobacco leaves and roots.

Starch was broken down into maltose by β-amylases (BAMs). In leaves before topping, the expression of BAM-encoding genes decreased with increasing nitrogen concentration. At the same nitrogen concentration, BAM expression was down-regulated after topping under low-nitrogen conditions but up-regulated after topping under medium- and high-

nitrogen conditions. In roots, BAM expression under low-nitrogen conditions was higher than under medium- and high-nitrogen conditions. These results indicate that topping enhances starch metabolism in tobacco leaves, whereas increasing nitrogen supply reduces starch metabolism in leaves. The gene encoding DPE2, which catalyzes the breakdown of maltose into glucose, was significantly up-regulated after topping in leaves under low-nitrogen conditions but down-regulated under medium- and high-nitrogen conditions after topping; in roots, it was up-regulated after topping. The hexokinase (HK)-encoding genes *Nta14g02740*, *Nta13g03640*, and *Nta07g25170* were up-regulated after topping in leaves, whereas the opposite pattern was observed in roots. In contrast, *Nta05g25850*, *Nta11g29290*, and *Nta06g28240* were up-regulated after topping in roots, indicating that hexokinase in roots and leaves is regulated by different homologous genes in response to topping. Genes encoding phosphoglucomutase (PGM) exhibited irregular changes under different nitrogen application rates and topping treatments. Two genes encoding phosphoglucose isomerase (PGI) showed opposite expression patterns in leaves after topping. Genes encoding sucrose phosphate synthase (SPS) and sucrose–phosphate phosphatase (SPP) in the sucrose synthesis pathway were up-regulated in leaves after topping. Genes encoding SUS, which catalyzes sucrose breakdown, showed no significant changes in leaves but were up-regulated in roots under medium- and high-nitrogen conditions. INV genes were down-regulated in leaves after topping but showed no significant changes in roots (Figure S8). These results indicate that under medium- and high-nitrogen conditions, topping up-regulated genes related to sucrose synthesis and down-regulated INV genes, thereby promoting sucrose accumulation.

In nitrogen metabolism, leaf NR-encoding genes showed decreased expression with increasing nitrogen application before topping, whereas their expression was up-regulated after topping at the same nitrogen concentration. In roots, NR expression increased with increasing nitrogen application; however, after topping at the same nitrogen concentration, NR was up-regulated only under low-nitrogen conditions, with no significant changes under medium- or high-nitrogen conditions. Leaf GS-encoding genes were up-regulated under low-nitrogen conditions and after topping, whereas in roots, no significant changes were observed except for up-regulation after topping under low-nitrogen conditions (Figure S8).

3.5.2. Effects of Different Nitrogen Application Rates and Topping Treatments on the Nicotine Biosynthetic Pathway

Oxaloacetate from the TCA cycle is converted to L-aspartate, which is then transformed into nicotinic acid via the NAD pathway under the catalysis of NAMNH (Figure 9). AO is a key enzyme in this process, and genes encoding AO were up-regulated in roots after topping under medium- and high-nitrogen conditions. Genes encoding A622 were up-regulated under medium-nitrogen conditions, whereas genes encoding other key enzymes in this pathway showed no significant changes under different nitrogen or topping treatments. N-Methylpyrrolinium is synthesized from L-ornithine and L-arginine, with ODC and ADC serving as the key enzymes. Genes encoding ODC were strongly induced under high-nitrogen conditions, while genes encoding ADC were highly expressed after topping under medium-nitrogen conditions and under high-nitrogen conditions (Figure S9). These results indicate that topping is the primary factor inducing high expression of AO-encoding genes, whereas nitrogen concentration is the primary factor inducing high expression of ODC- and ADC-encoding genes.

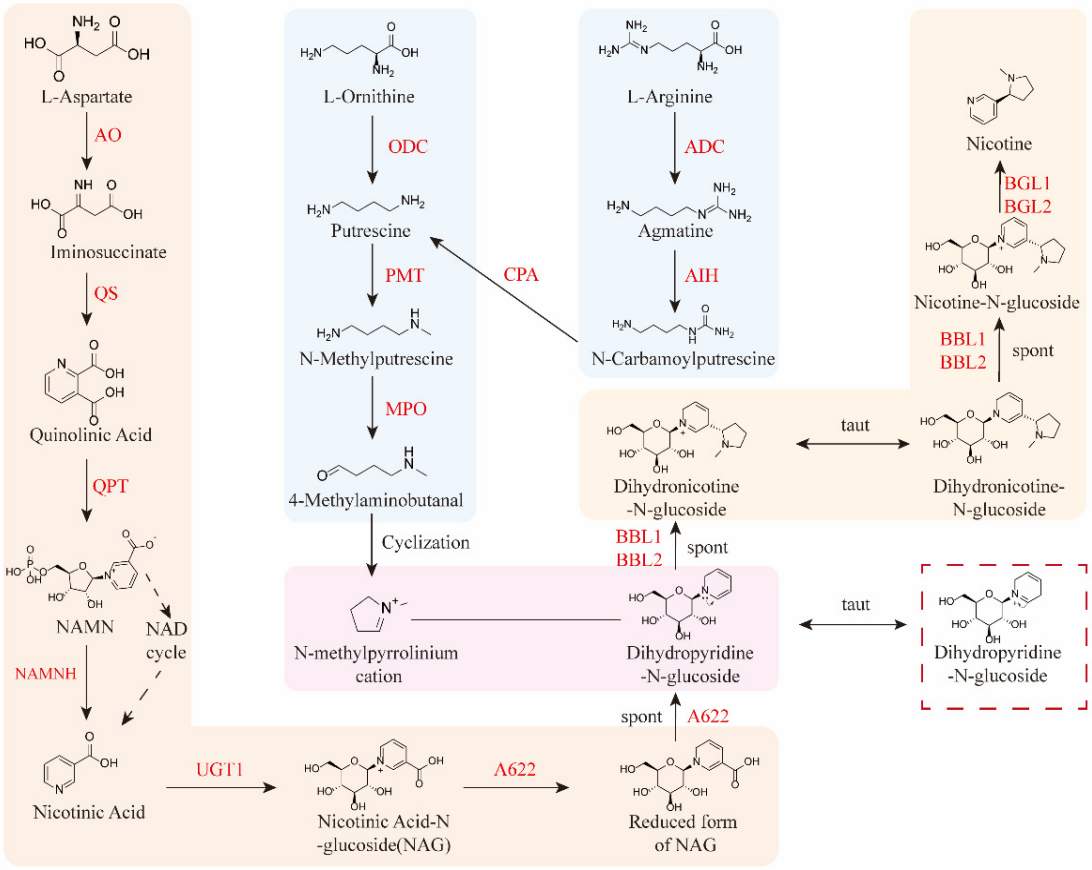


Figure 9. Effects of different nitrogen application rates and topping treatments on nicotine metabolism.

4. Discussion

4.1. Stage-Dependent Regulation of Agronomic Traits by Different Nitrogen Application Rates

In this study, the regulation of tobacco agronomic traits by nitrogen supply exhibited pronounced stage-dependent characteristics. During the rosette and vigorous growing stages, these agronomic traits increased significantly with increasing nitrogen application (Figure 1I–VI). Nitrogen is a critical component of chlorophyll, phytohormones, amino acids, nucleic acids, and secondary metabolites and is a major factor influencing photosynthesis, indicating that nitrogen is the primary limiting factor for photosynthetic capacity and biomass accumulation during the vigorous growth phase of tobacco [35,36]. However, after entering the budding stage, although traits such as plant height and stem diameter continued to respond to nitrogen concentration, fifth-leaf length and width no longer differed significantly between the medium- and high-nitrogen treatments. This suggests that leaf sensitivity to nitrogen may decline after the budding stage, with more nitrogen translocated to reproductive organs or allocated to secondary metabolism. This phenomenon has been verified in *Arabidopsis* and oilseed rape, in which nitrogen can be remobilized from senescing leaves and transported to organs during the vegetative and reproductive growth stages [37–39]. Therefore, maintaining a medium nitrogen level before the budding stage is sufficient to achieve high yield; continued fertilization beyond this level not only fails to further expand leaf area but may also lead to decreased soil pH and soil acidification [40]. Correlation analysis between soil pH and agronomic traits further supported this conclusion, as soil pH was negatively correlated with agronomic traits as nitrogen application increased at the budding stage. Although plant height, stem diameter, and effective leaf number continued to increase with higher nitrogen rates at the budding stage (Figure 1), excessive vegetative growth is not desirable for flue-cured tobacco. Through

continuous observation across the three growth stages, this study identified the rosette and vigorous growing stages as the critical periods for nitrogen fertilizer input. Considering the comprehensive effects on agronomic traits, leaf quality, and soil environment, a medium nitrogen level is optimal for achieving high yield without inducing excessive vegetative growth or soil acidification.

4.2. Differential Regulation of Tobacco Leaf Quality by Different Nitrogen Application Rates

The effects of different nitrogen application rates on tobacco leaf quality exhibited trait-specific responses, with interactive effects between leaf position and nitrogen concentration. At the same leaf position, both total nitrogen and total alkaloid contents increased with increasing nitrogen application; however, total alkaloid content did not differ significantly between the medium- and high-nitrogen treatments. This suggests the existence of a saturation threshold for total alkaloid synthesis in tobacco leaves. When nitrogen concentration exceeds the medium level, total alkaloid content may continue to increase with rising nitrogen supply but no longer differs significantly from that under medium-nitrogen conditions, as nitrogen is preferentially allocated to amino acids, proteins [41], and other compounds, while also coordinating root and other organ growth [42,43]. Total nitrogen content in upper leaves was higher than that in middle and lower leaves, which may be attributed to the fact that upper leaves serve as the primary sites for active growth, metabolism, and photosynthesis [16], consistent with previous studies showing greater nitrogen uptake in upper leaves than in lower leaves [44]. Total sugar content decreased with increasing nitrogen concentration, a trend closely linked to carbon–nitrogen metabolism [45–47] and consistent with studies showing that nitrogen availability affects organic acid and carbohydrate synthesis in plants [48,49]. Potassium and chloride contents in tobacco leaves have important implications for leaf quality [50]. In this study, potassium content decreased with increasing nitrogen application, likely due to competitive uptake and transport between NH_4^+ and K^+ [51]. A decrease in K^+ influx in the presence of NH_4^+ has been observed in various crops [52,53]. Chloride content was lowest under medium-nitrogen conditions across all leaf positions. Therefore, appropriate nitrogen application helps reduce chloride content, maintain reasonable potassium levels, and improve leaf quality and combustibility. Considering all quality indicators comprehensively, although the medium-nitrogen treatment reduced total sugar and reducing sugar contents compared with the low-nitrogen treatment, it enhanced the coordination between total alkaloids and total nitrogen while optimizing the potassium-to-chloride ratio, thereby more effectively improving tobacco leaf quality.

4.3. Associations Between Soil Physicochemical Properties and Tobacco Leaf Quality Under Different Nitrogen Application Rates

In this study, soil pH exhibited an initial increase followed by a decrease as growth progressed. At the rosette and vigorous growing stages, pH increased with increasing nitrogen application, whereas the opposite trend occurred at the budding stage. This may be attributed to the high nitrogen demand during vegetative growth, when nitrogen is taken up predominantly as nitrate [23]. Nitrate transport requires co-transport with H^+ into root cells, thereby alkalizing the rhizosphere [54,55]. Upon entering the budding stage, tobacco transitions to reproductive growth, nitrate demand decreases, and nitrification of ammonium ions may contribute to soil acidification. Recent studies have reported that under acidified conditions, viruses can influence soil phosphorus accumulation by modulating prokaryotic phosphate cycling processes, consistent with the highest phosphorus content observed under high-nitrogen conditions in this study [56]. Correlation analysis between soil physicochemical properties and leaf quality revealed dynamic relationships

across growth stages. At the rosette stage, soil pH, total nitrogen, hydrolyzable nitrogen, organic matter, and available phosphorus were positively correlated with agronomic traits. However, these soil properties were negatively correlated with leaf quality indicators such as potassium, reducing sugar, and total sugar contents, but positively correlated with total alkaloid and total nitrogen contents. This indicates that soil fertility at the rosette stage establishes the baseline for subsequent quality formation, wherein high fertility promotes vegetative growth and nitrogen accumulation but suppresses sugar and potassium accumulation. At the vigorous growing stage, available potassium emerged as a key factor, positively correlated with potassium, reducing sugar, and total sugar contents, and negatively correlated with total alkaloid and chloride contents. This may be because the vigorous growing stage is a period of rapid enhancement of photosynthetic capacity in leaves, during which potassium, as a stomatal regulator and enzyme activator [57,58], influences carbon assimilation and sugar accumulation.

The soil organic matter (SOM) content under the medium-nitrogen treatment was significantly higher than that under the low- and high-nitrogen treatments, which is consistent with previous related studies [59,60]. The ratio of fungi to bacteria (F:B ratio) has a significant impact on soil organic matter (SOM) [61]; fungal-dominated soils can sequester more carbon and retain more nitrogen [62]. Previous studies have shown that under high nitrogen input, the soil microbial community shifts towards bacterial dominance, and the relatively stronger bacterial metabolism consumes soil organic carbon. However, optimized nitrogen application promotes an increase in the proportion and activity of soil fungi, thereby facilitating long-term carbon storage [60]. In contrast, under no nitrogen application, microbial growth is limited due to nitrogen deficiency [63]. In our study, SOM was calculated from SOC (SOM = SOC × 1.724); therefore, we inferred that the medium-nitrogen treatment could promote organic matter turnover. Furthermore, both soil chloride content and mature leaf chloride content under the high-nitrogen level were higher than those under the low- and medium-nitrogen levels. However, the specific interaction between nitrogen application rate and rhizosphere chloride dynamics has rarely been reported; hence, the underlying mechanisms warrant further investigation.

4.4. Molecular Mechanisms Underlying the Synergistic Effects of Medium Nitrogen and Topping on Tobacco Leaf Quality

The combination of medium nitrogen and topping exerted the most profound effects on both the transcriptome and metabolome, optimizing the carbon–nitrogen metabolic balance and nicotine metabolism, thereby improving tobacco leaf quality. After topping, differentially expressed genes were mainly enriched in amino acid metabolism and energy metabolism, while differentially accumulated metabolites were predominantly amino acids, alkaloids, and terpenoids.

With respect to nitrogen metabolism, topping significantly increased the activities of nitrate reductase (NR) and glutamine synthetase (GS), as well as the expression of their corresponding genes in leaves across all nitrogen levels, while significantly reducing free ammonia content. Notably, free ammonia content was highest under low-nitrogen conditions, which may be attributed to NH_4^+ production via protein degradation under nitrogen deficiency, impaired chloroplast function, insufficient carbon skeletons, and reduced GS activity, collectively leading to excessive NH_4^+ accumulation [64,65]. Topping promoted nitrogen metabolism by converting free ammonia into nitrogen-containing compounds such as nicotine. Although NR and GS activities were highest under high-nitrogen conditions, GS activity did not differ significantly between the medium- and high-nitrogen treatments. The assimilation of NH_4^+ into glutamine requires the carbon skeleton α-ketoglutarate [66]; therefore, under high-nitrogen conditions, NH_4^+ assimilation may be constrained by an

insufficient carbon skeleton supply. Under medium-nitrogen conditions, free ammonia content was lowest, indicating that tobacco plants utilized free ammonia most efficiently under this regime.

With respect to carbon metabolism, topping significantly increased sucrose phosphate synthase (SPS) activity and sucrose content only under medium-nitrogen conditions, whereas this effect was absent under low- and high-nitrogen conditions. Meanwhile, starch content decreased significantly after topping across all nitrogen levels, while total sugar and sucrose contents increased significantly. Consistent with the transcriptomic data, topping up-regulated the expression of genes encoding sucrose synthesis-related enzymes (*SPS* and *SPP*) under medium- and high-nitrogen conditions while down-regulating invertase (*INV*) gene expression. The rationale underlying these carbon metabolic changes may lie in the enhanced nitrogen metabolism following topping, which demands substantial energy and carbon skeletons [66]. Plants respond by degrading leaf starch and converting it into transportable, utilizable soluble sugars such as sucrose, thereby supporting the synthesis of secondary metabolites, including nicotine. Additionally, starch degradation after topping may enhance photosynthesis; previous studies have shown that excessive starch can compress and damage photosynthetic membrane structures, leading to reduced photosynthetic efficiency [67]. After topping, tobacco leaves exhibited fewer and smaller starch granules, as well as increased stomatal density and number, thereby enhancing photosynthesis and promoting sugar accumulation [68]. Insufficient sugar content in tobacco leaves results in harsh, irritating smoke, whereas excessive sugar content leads to bland, insipid smoke [69]. Under medium-nitrogen conditions, both the overconsumption of carbon skeletons that reduces total sugar content under high nitrogen and the insufficient nitrogen supply and restricted nicotine synthesis observed under low nitrogen were avoided.

Nicotine is the most important secondary metabolite in tobacco, playing a pivotal role in stimulating the nervous system and inducing pleasurable sensations in smokers. The promoting effect of topping on nicotine biosynthesis is achieved through multi-pathway, multi-organ synergistic transcriptional reprogramming [70]. Topping has been shown to up-regulate the expression of genes encoding nicotine biosynthesis-related enzymes, such as *PMT* [71]. In the present study, genes encoding *AO* in roots were up-regulated after topping under medium- and high-nitrogen conditions, and genes encoding *A622* were up-regulated under medium-nitrogen conditions, whereas genes encoding *ODC* and *ADC* were primarily influenced by nitrogen concentration. No significant changes were observed in the expression of other genes encoding enzymes involved in the nicotine biosynthetic pathway, which may be attributed to factors such as sampling time, at which point their expression levels may no longer show significant alterations.

4.5. Integrated Analysis of Transcriptomic and Metabolomic Data Under Different Nitrogen Treatments and After Topping

Topping promotes root development, enhances root vitality, and increases nicotine synthesis capacity [72]. Weighted gene co-expression network analysis (WGCNA) is a method for dissecting correlations between gene co-expression networks and target traits based on transcriptomic data [73]. Through WGCNA, the Pfam annotations of genes in the module most highly correlated with nicotine content were predominantly cytochrome P450, Terpene_synth, and Terpene_synth_C. Previous studies have demonstrated that cytochrome P450 genes are involved in terpenoid biosynthesis [74], the biosynthesis of plant diterpenoid natural products, and alkaloid biosynthesis [75]; however, no direct association with nicotine synthesis has been reported to date. Moreover, cytochrome P450s (CYPs) play critical roles in plant stress adaptation. They are involved in hormone synthesis and metabolite catabolism, helping plants resist biotic and abiotic stresses and

enhancing their resistance and adaptability. For example, overexpression of *FtCYP94C1* in *Arabidopsis thaliana* enhanced disease resistance [76], and topping itself represents a form of stress in tobacco. Therefore, genes annotated as cytochrome P450, Terpene_synth, and Terpene_synth_C in the darkturquoise module may have functions that directly or indirectly regulate nicotine biosynthesis.

5. Conclusions

This study, through integrated analyses of agronomy, soil science, and metabolomics/multi-omics, demonstrated that a medium nitrogen application rate (120 kg/ha) combined with topping is the optimal strategy for enhancing tobacco quality. This combination optimized the chemical composition of tobacco leaves by enhancing carbon and nitrogen metabolism, promoting the conversion of starch to soluble sugars, and alleviating ammonium–potassium competition and soil acidification, while eliciting the most pronounced responses at both the transcriptomic and metabolomic levels. Weighted gene co-expression network analysis (WGCNA) identified modules highly correlated with nicotine biosynthesis, with hub genes predominantly belonging to the cytochrome P450 and terpene synthase families, providing support for the molecular regulation of nicotine synthesis. However, this study has the following limitations: transcriptomic and metabolomic analyses were performed only at 10 days after topping, which did not capture the complete dynamic process; the candidate hub genes have not yet been functionally validated; and all plants were topped, with no omics analysis of untopped plants at the same stage after topping. Future research should focus on (1) functional validation of candidate genes via genetic transformation; (2) dynamic omics analysis at multiple time points after topping, as well as omics analysis of untopped plants at the same stage after topping; and (3) multi-site, multi-year field validation trials.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agronomy16212171/s1. Figure S1: Physicochemical properties of tobacco rhizosphere soil at different periods under different nitrogen application rates; Figure S2: Enrichment analysis of the merged differentially expressed genes of NBLU and NTLU; Figure S3: Enrichment analysis of the merged differentially expressed genes of NBRU and TRU; Figure S4: GO enrichment analysis (A) and KEGG enrichment analysis (B) of the merged differentially expressed genes of NTRU; Figure S5: K-means clustering analysis of the merged differentially abundant metabolites of NBLU, NBRU, NTLU, and NTRU and metabolite statistics after clustering; Figure S6: K-means clustering analysis of the merged differentially abundant metabolites of TLU and TRU, with the statistical results of metabolites after clustering displayed; Figure S6: WGCNA analysis of metabolites; Figure S8: Heatmap of the expression levels of genes encoding carbon and nitrogen metabolism enzymes in roots and leaves; Figure S9: Heatmap of the expression levels of genes encoding nicotine metabolism-related enzymes in roots and leaves; Table S1: Analysis of quality traits of mature leaves at different positions under different nitrogen application rates; Table S2: Effect of topping on nitrogen metabolism at different nitrogen application rates; Table S3: Effects of topping on carbon metabolism under different nitrogen application rates; Table S4: Physicochemical properties of tobacco rhizosphere soil at different periods under different nitrogen application rates; Table S5: Transcriptome sequencing quality metrics; Table S6: Detailed information of GO enrichment analysis of the merged differentially expressed genes of NBLU; Table S7: Detailed information of GO enrichment analysis of the merged differentially expressed genes of NBRU; Table S8: Detailed information of GO enrichment analysis of the merged differentially expressed genes of NTLU; Table S9: Detailed information of GO enrichment analysis of the merged differentially expressed genes of NTRU; Table S10: Detailed information of GO enrichment analysis of the merged differentially expressed genes of TLU; Table S11: Detailed information of GO enrichment analysis of the merged differentially expressed genes of TRU; Table S12: Detailed information of KEGG enrichment pathways of the merged differentially expressed genes of NBLU; Table S13: Detailed information of KEGG enrichment pathways of the

merged differentially expressed genes of NBRU; Table S14: Detailed information of KEGG enrichment pathways of the merged differentially expressed genes of NTLU; Table S15: Detailed information of KEGG enrichment pathways of the merged differentially expressed genes of NTRU; Table S16: Detailed information of KEGG enrichment pathways of the merged differentially expressed genes of TLU; Table S17: Detailed information of KEGG enrichment pathways of the merged differentially expressed genes of TRU; Table S18: Detailed information of metabolites in metabolomics; Table S19: Summary table of differentially abundant metabolites; Table S20: Gene annotation information of the darkturquoise module.

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Abbreviations

The following abbreviations are used in this manuscript:

NRTs	nitrate transporters
NR	nitrate reductase
NiR	nitrite reductase
NH_4^+	ammonium
Gln	glutamine
Glu	glutamate
GS	glutamine synthetase
GOGAT	glutamate synthase
K^+	potassium
LN	low nitrogen
MN	medium nitrogen
HN	high nitrogen
PH	Soil pH
SOM	soil organic matter
TN	total nitrogen
AN	alkali-hydrolyzable nitrogen
AP	available phosphorus
AK	available potassium
WSC	water-soluble chloride
TCA	trichloroacetic acid
SPS	sucrose phosphate synthase
SUS	sucrose synthase
MWDB	MetWare Database
DAMs	Differentially accumulated metabolites
VIP	Variable Importance in Projection
TPM	Transcripts per million
DEGs	differentially expressed genes
PCA	principal component analysis
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes

ANOVA	one-way analysis of variance
Sd	stem diameter
Ph	plant height
Nel	number of effective leaves
Ll	fifth-leaf length
Il	internode length
Wl	fifth-leaf width
RS	reducing sugar
TS	total sugar content
TA	total alkaloid
Tn	leaf total nitrogen
K	leaf potassium
Cl	chloride
FA	free ammonia content
StC	starch content
SC	sucrose content
Nic	nicotine content
BP	Biological Process
MF	Molecular Function
NBLU	denotes the union set of DEGs in leaves in response to increasing nitrogen application before topping
NBRU	denotes the union set in roots in response to increasing nitrogen application before topping
NTLU	denotes the union set in leaves in response to increasing nitrogen application after topping
NTRU	denotes the union set in roots in response to increasing nitrogen application after topping
TLU	denotes the union set of DEGs in leaves after topping at the same nitrogen concentration
TRU	denotes the union set in roots after topping at the same nitrogen concentration
BAMs	β-amylases
DPE2	disproportionating enzyme 2
HK	hexokinase
PGM	phosphoglucomutase
PGI	phosphoglucose isomerase
SPP	sucrose phosphate phosphatase
INV	invertase
OAT	ornithine δ-aminotransferase
AO	L-aspartate oxidase
QS	quinolinate synthase
A622	isoflavone reductase-like protein A622
BBL	berberine bridge enzyme-like protein
ODC	ornithine decarboxylase
PMT	putrescine N-methyltransferase
UGT1	UDP-glycosyltransferase
MPO	N-methylputrescine oxidase
ADC	arginine decarboxylase
CPA	N-carbamoylputrescine amidohydrolase
BGL	β-glucosidase
NAD	nicotinamide adenine dinucleotide pathway
WGCNA	Weighted gene co-expression network analysis
CYPs	cytochrome P450s
IQR	interquartile range

References

1. Kole, C. *Wild Crop Relatives: Genomic and Breeding Resources: Legume Crop and Forages*; Springer: Berlin/Heidelberg, Germany, 2011.
2. Kurt, D. Impacts of Environmental Variations on Quality and Chemical Contents of Oriental Tobacco. *Contrib. Tob. Nicotine Res.* **2021**, *30*, 50–62. [CrossRef]
3. Xia, B.; Feng, M.; Xu, G.; Xu, J.; Li, S.; Chen, X.; Ding, L.; Zhou, Y. Investigation of the chemical compositions in tobacco of different origins and maturities at harvest by GC-MS and HPLC-PDA-QTOF-MS. *J. Agric. Food Chem.* **2014**, *62*, 4979–4987. [CrossRef]
4. Talhout, R.; Opperhuizen, A.; van Amsterdam, J.G.C. Sugars as tobacco ingredient: Effects on mainstream smoke composition. *Food Chem. Toxicol.* **2006**, *44*, 1789–1798. [CrossRef]

5. Roemer, E.; Schorp, M.K.; Piadé, J.J.; Seeman, J.I.; Leyden, D.E.; Haussmann, H.J. Scientific assessment of the use of sugars as cigarette tobacco ingredients: A review of published and other publicly available studies. *Crit. Rev. Toxicol.* **2012**, *42*, 244–278. [CrossRef]
6. Niu, L.; Hu, W.; Wang, F.; Shaker, M.; Yang, X. Integrated transcriptomic and metabolomic analyses elucidate the mechanism by which grafting impacts potassium utilization efficiency in tobacco. *BMC Plant Biol.* **2025**, *25*, 94. [CrossRef]
7. Meng, X.; Wang, X.; Zhang, Z.; Xiong, S.; Wei, Y.; Guo, J.; Zhang, J.; Wang, L.; Ma, X.; Tegeder, M. Transcriptomic, proteomic, and physiological studies reveal key players in wheat nitrogen use efficiency under both high and low nitrogen supply. *J. Exp. Bot.* **2021**, *72*, 4435–4456. [CrossRef]
8. Sifola, M.I.; Carrino, L.; Cozzolino, E.; Ianuario, S.; Lucibelli, A.; Coppola, A. A Survey of Fertility Program Responses of Kentucky Dark Fire-Cured Tobacco (*Nicotiana tabacum* L.) Yield and Quality for Cigars Manufacture in the Benevento Province (Southern Italy). *Contrib. Tob. Nicotine Res.* **2018**, *28*, 14–29. [CrossRef]
9. Zhang, B.; Chen, T.; Wang, B. Effects of Long-term Uses of Chemical Fertilizers on Soil Quality. *Chin. Agric. Sci. Bull.* **2010**, *26*, 182–287. Available online: https://www.casb.org.cn/EN/Y2010/V26/I11/182 (accessed on 17 October 2026).
10. O'Brien, J.A.; Vega, A.; Bouguyon, E.; Krouk, G.; Gojon, A.; Coruzzi, G.; Gutiérrez, R.A. Nitrate Transport, Sensing, and Responses in Plants. *Mol. Plant* **2016**, *9*, 837–856. [CrossRef]
11. Tegeder, M.; Masclaux-Daubresse, C. Source and sink mechanisms of nitrogen transport and use. *New Phytol.* **2018**, *217*, 35–53. [CrossRef]
12. Wu, X.; Zhou, X.; Wang, S.; Wang, Z.; Huang, P.; Pu, W.; Peng, Y.; Fan, X.; Gao, J.; Li, Z. Overexpression of a nitrate transporter NtNPF2.11 increases nitrogen accumulation and yield in tobacco. *Gene* **2023**, *885*, 147715. [CrossRef]
13. Shoji, T. Nicotine Biosynthesis, Transport, and Regulation in Tobacco: Insights into the Evolution of a Metabolic Pathway. In *The Tobacco Plant Genome*; Ivanov, N.V., Sierro, N., Peitsch, M.C., Eds.; Springer International Publishing: Cham, Switzerland, 2020; pp. 147–156.
14. Dewey, R.E.; Xie, J. Molecular genetics of alkaloid biosynthesis in *Nicotiana tabacum*. *Phytochemistry* **2013**, *94*, 10–27. [CrossRef]
15. Chang, L.; Xu, Z.; Deng, P.; Zhang, N.; He, T.; Liu, X.; He, W.; Zheng, A.; Hu, W.; Pan, M.; et al. Complete biosynthesis of nicotine. *Cell* **2026**, *189*, 2700–2713.e2720. [CrossRef]
16. Lei, B.; Chang, W.; Zhao, H.; Zhang, K.; Yu, J.; Yu, S.; Cai, K.; Zhang, J.; Lu, K. Nitrogen application and differences in leaf number retained after topping affect the tobacco (*Nicotiana tabacum*) transcriptome and metabolome. *BMC Plant Biol.* **2022**, *22*, 38. [CrossRef]
17. Singh, S.K.; Wu, Y.; Ghosh, J.S.; Pattanaik, S.; Fisher, C.; Wang, Y.; Lawson, D.; Yuan, L. RNA-sequencing Reveals Global Transcriptomic Changes in *Nicotiana tabacum* Responding to Topping and Treatment of Axillary-shoot Control Chemicals. *Sci. Rep.* **2015**, *5*, 18148. [CrossRef]
18. Gu, K.; Yang, L.E.; Ren, K.; Luo, X.; Qin, X.; Op de Beeck, M.; He, C.; Jian, L.; Chen, Y. Effects of topping and non-topping on growth-regulating hormones of flue-cured tobacco (*Nicotiana tabacum* L.)—A proteomic analysis. *Front. Plant Sci.* **2023**, *14*, 1255252. [CrossRef]
19. Yan, S.; Niu, Z.; Yan, H.; Zhang, A.; Liu, G. Transcriptome sequencing reveals the effect of biochar improvement on the development of tobacco plants before and after topping. *PLoS ONE* **2019**, *14*, e0224556. [CrossRef]
20. Liao, R.; Dongchen, W.; Mao, Z.; Wang, C.; Xiong, Y.; Geng, S.; Lin, C.; Liu, Z. Novel winter crop integration enhances crop productivity, economic benefits, and their stability in tobacco-based paddy-upland rotations under climate-driven dynamics. *Field Crops Res.* **2026**, *345*, 110544. [CrossRef]
21. Liao, R.; Dongchen, W.; Lin, C.; Zhou, S.; Dong, Y.; Geng, C.; Liu, Z.; Mao, Z. Precipitation-driven rhizosphere microbiome regulates tobacco leaf irritancy via carbon–nitrogen metabolic coordination. *Chem. Biol. Technol. Agric.* **2025**, *12*, 172. [CrossRef]
22. Luo, J.; Hang, J.; Wu, B.; Wei, X.; Zhao, Q.; Fang, Z. Co-overexpression of genes for nitrogen transport, assimilation, and utilization boosts rice grain yield and nitrogen use efficiency. *Crop J.* **2023**, *11*, 785–799. [CrossRef]
23. Miao, C.; Hu, Z.; Ye, H.; Liu, X.; Wang, Y.; Tan, J.; Jiang, H.; Chen, J. Overexpression of a high affinity nitrate transporter NtNRT2.1 significantly improves the nicotine content in *Nicotiana tabacum*. *Ind. Crops Prod.* **2025**, *224*, 120326. [CrossRef]
24. Yan, Y.; Zhang, Z.; Sun, H.; Liu, X.; Xie, J.; Qiu, Y.; Chai, T.; Chu, C.; Hu, B. Nitrate confers rice adaptation to high ammonium by suppressing its uptake but promoting its assimilation. *Mol. Plant* **2023**, *16*, 1871–1874. [CrossRef]
25. Chen, W.; Gong, L.; Guo, Z.; Wang, W.; Zhang, H.; Liu, X.; Yu, S.; Xiong, L.; Luo, J. A novel integrated method for large-scale detection, identification, and quantification of widely targeted metabolites: Application in the study of rice metabolomics. *Mol. Plant* **2013**, *6*, 1769–1780. [CrossRef]
26. Wang, X.; Chai, X.; Gao, B.; Deng, C.; Günther, C.S.; Wu, T.; Zhang, X.; Xu, X.; Han, Z.; Wang, Y. Multi-omics analysis reveals the mechanism of bHLH130 responding to low-nitrogen stress of apple rootstock. *Plant Physiol.* **2023**, *191*, 1305–1323. [CrossRef]
27. Kim, D.; Paggi, J.M.; Park, C.; Bennett, C.; Salzberg, S.L. Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. *Nat. Biotechnol.* **2019**, *37*, 907–915. [CrossRef]

28. Wang, J.; Zhang, Q.; Tung, J.; Zhang, X.; Liu, D.; Deng, Y.; Tian, Z.; Chen, H.; Wang, T.; Yin, W.; et al. High-quality assembled and annotated genomes of *Nicotiana tabacum* and Nicotiana benthamiana reveal chromosome evolution and changes in defense arsenals. *Mol. Plant* **2024**, *17*, 423–437. [CrossRef]
29. Cantalapiedra, C.P.; Hernández-Plaza, A.; Letunic, I.; Bork, P.; Huerta-Cepas, J. eggNOG-mapper v2: Functional Annotation, Orthology Assignments, and Domain Prediction at the Metagenomic Scale. *Mol. Biol. Evol.* **2021**, *38*, 5825–5829. [CrossRef]
30. Love, M.I.; Huber, W.; Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **2014**, *15*, 550. [CrossRef]
31. Ashburner, M.; Ball, C.A.; Blake, J.A.; Botstein, D.; Butler, H.; Cherry, J.M.; Davis, A.P.; Dolinski, K.; Dwight, S.S.; Eppig, J.T.; et al. Gene ontology: Tool for the unification of biology. *Nat. Genet.* **2000**, *25*, 25–29. [CrossRef]
32. Groza, T.; Marcello, A.J.; Carlisle, T.; Lim, W.K.; Haendel, M.; Karnani, N.; Robinson, P.N.; Graessner, H.; Chong, J.X.; Baynam, G.; et al. A systematic assessment of large language models' knowledge of rare diseases: How much do large language models know about rare disease? *HGG Adv.* **2026**, *7*, 100558. [CrossRef]
33. Ogata, H.; Goto, S.; Sato, K.; Fujibuchi, W.; Bono, H.; Kanehisa, M. KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Res.* **1999**, *27*, 29–34. [CrossRef]
34. Wu, T.; Hu, E.; Xu, S.; Chen, M.; Guo, P.; Dai, Z.; Feng, T.; Zhou, L.; Tang, W.; Zhan, L.; et al. clusterProfiler 4.0: A universal enrichment tool for interpreting omics data. *Innovation* **2021**, *2*, 100141. [CrossRef]
35. Kraiser, T.; Gras, D.E.; Gutiérrez, A.G.; González, B.; Gutiérrez, R.A. A holistic view of nitrogen acquisition in plants. *J. Exp. Bot.* **2011**, *62*, 1455–1466. [CrossRef]
36. Wang, L.; Chen, S.; Shi, W.; Xing, S.; Han, C.; Bai, M.Y.; Fan, M. Nitrate starvation inhibits stomatal opening via the long-distance CEP1-CEPR2 signaling cascade. *Cell Rep.* **2025**, *44*, 116424. [CrossRef]
37. Malagoli, P.; Laine, P.; Rossato, L.; Ourry, A. Dynamics of nitrogen uptake and mobilization in field-grown winter oilseed rape (Brassica napus) from stem extension to harvest: I. Global N flows between vegetative and reproductive tissues in relation to leaf fall and their residual N. *Ann. Bot.* **2005**, *95*, 853–861. [CrossRef]
38. Diaz, C.; Lemaître, T.; Christ, A.; Azzopardi, M.; Kato, Y.; Sato, F.; Morot-Gaudry, J.F.; Le Dily, F.; Masclaux-Daubresse, C. Nitrogen recycling and remobilization are differentially controlled by leaf senescence and development stage in Arabidopsis under low nitrogen nutrition. *Plant Physiol.* **2008**, *147*, 1437–1449. [CrossRef]
39. Masclaux-Daubresse, C.; Daniel-Vedele, F.; Dechorgnat, J.; Chardon, F.; Gaufichon, L.; Suzuki, A. Nitrogen uptake, assimilation and remobilization in plants: Challenges for sustainable and productive agriculture. *Ann. Bot.* **2010**, *105*, 1141–1157. [CrossRef]
40. Hao, T.; Zhu, Q.; Zeng, M.; Shen, J.; Shi, X.; Liu, X.; Zhang, F.; de Vries, W. Impacts of nitrogen fertilizer type and application rate on soil acidification rate under a wheat-maize double cropping system. *J. Environ. Manag.* **2020**, *270*, 110888. [CrossRef]
41. Li, S.; Ahmed, W.; Jiang, T.; Yang, D.; Yang, L.; Hu, X.; Zhao, M.; Peng, X.; Yang, Y.; Zhang, W.; et al. Amino acid metabolism pathways as key regulators of nitrogen distribution in tobacco: Insights from transcriptome and WGCNA analyses. *BMC Plant Biol.* **2025**, *25*, 393. [CrossRef]
42. Shen, C.; Ji, Z.; Jiao, W.; Zhang, S.; Huang, Y.; Qin, Y.; Huang, M.; Kang, S.; Mo, Z.; Jiang, B.; et al. OsWRI1a coordinates systemic growth responses to nitrogen availability in rice. *Science* **2026**, *391*, 937–945. [CrossRef]
43. Xu, G.; Fan, X.; Miller, A.J. Plant nitrogen assimilation and use efficiency. *Annu. Rev. Plant Biol.* **2012**, *63*, 153–182. [CrossRef]
44. Minjue, C.; Jianan, Z.; Ruiyin, H.; Xianyun, Z.; Shuai, Z.; Hongxing, L.; Xu, W.; Jianjun, C.; Yuanyuan, W. Quantitative relationships between nitrogen fertilizer use efficiency and contents of alkaloid components in flue-cured tobacco. *Tob. Sci. Technol.* **2025**, *58*, 34–45. [CrossRef]
45. Nunes-Nesi, A.; Fernie, A.R.; Stitt, M. Metabolic and signaling aspects underpinning the regulation of plant carbon nitrogen interactions. *Mol. Plant* **2010**, *3*, 973–996. [CrossRef]
46. Bi, Y.M.; Wang, R.L.; Zhu, T.; Rothstein, S.J. Global transcription profiling reveals differential responses to chronic nitrogen stress and putative nitrogen regulatory components in Arabidopsis. *BMC Genom.* **2007**, *8*, 281. [CrossRef]
47. Krapp, A.; Berthomé, R.; Orsel, M.; Mercey-Boutet, S.; Yu, A.; Castaings, L.; Elftieh, S.; Major, H.; Renou, J.P.; Daniel-Vedele, F. Arabidopsis roots and shoots show distinct temporal adaptation patterns toward nitrogen starvation. *Plant Physiol.* **2011**, *157*, 1255–1282. [CrossRef]
48. Tian, J.; Pang, Y.; Yuan, W.; Peng, J.; Zhao, Z. Growth and nitrogen metabolism in *Sophora japonica* (L.) as affected by salinity under different nitrogen forms. *Plant Sci.* **2022**, *322*, 111347. [CrossRef]
49. Li, X.; Yao, H.; Wang, G.; Wang, N.; Luo, Q.; Zhao, G.; Wu, Y.; Zhang, G.; Duan, X.; Bai, Y.; et al. Integrated plant physiology, transcriptomics, and metabolomics reveal tobacco root growth and nicotine synthesis responses in tobacco exposure to different nitrogen forms. *Chem. Biol. Technol. Agric.* **2025**, *12*, 38. [CrossRef]
50. Chen, D.; Chen, H.; Sun, H.; Man, Y.; Long, J.; Liu, M.; Cai, J.; Li, D.; Xu, S.; Liu, K.; et al. Physicochemical properties of three Yunnan cigar tobacco leaves and identification of aroma compounds in tobacco smoke. *Front. Chem.* **2025**, *13*, 1664073. [CrossRef]
51. Coskun, D.; Britto, D.T.; Kronzucker, H.J. The nitrogen-potassium intersection: Membranes, metabolism, and mechanism. *Plant Cell Environ.* **2017**, *40*, 2029–2041. [CrossRef]

52. Zhang, F.; Niu, J.; Zhang, W.; Chen, X.; Li, C.; Yuan, L.; Xie, J. Potassium nutrition of crops under varied regimes of nitrogen supply. *Plant Soil* **2010**, *335*, 21–34. [CrossRef]
53. Szczerba, M.W.; Britto, D.T.; Kronzucker, H.J. K+ transport in plants: Physiology and molecular biology. *J. Plant Physiol.* **2009**, *166*, 447–466. [CrossRef]
54. Rivero-Marcos, M.; Lasa, B.; Neves, T.; Zamarreño, Á.M.; García-Mina, J.M.; García-Olaverri, C.; Aparicio-Tejo, P.M.; Cruz, C.; Ariz, I. Plant ammonium sensitivity is associated with external pH adaptation, repertoire of nitrogen transporters, and nitrogen requirement. *J. Exp. Bot.* **2024**, *75*, 3557–3578. [CrossRef]
55. Feng, H.; Fan, X.; Miller, A.J.; Xu, G. Plant nitrogen uptake and assimilation: Regulation of cellular pH homeostasis. *J. Exp. Bot.* **2020**, *71*, 4380–4392. [CrossRef]
56. Cheng, S.; Dai, Z.; He, D.; Xue, R.; Zhao, K.; Mazza Rodrigues, J.L.; Mendes, L.W.; Chang, S.X.; Xu, J. Long-term N fertilization altered viral community composition and increased functional potentials of phosphorus cycling by causing soil acidification. *Soil Biol. Biochem.* **2026**, *217*, 110137. [CrossRef]
57. Alvim, F.; Alvim, J.C.; Hibberd, J.M.; Harvey, A.R.; Blatt, M.R. A C4 plant K+ channel accelerates stomata to enhance C3 photosynthesis and water use efficiency. *Plant Physiol.* **2025**, *197*, kiaf039. [CrossRef]
58. Feng, X.; Liu, W.; Cao, F.; Wang, Y.; Zhang, G.; Chen, Z.H.; Wu, F. Overexpression of HvAKT1 improves drought tolerance in barley by regulating root ion homeostasis and ROS and NO signaling. *J. Exp. Bot.* **2020**, *71*, 6587–6600. [CrossRef]
59. Zhang, X.; Han, X.; Li, Q.; Chen, Q.; Wu, Z.; Zhao, J.; Zhang, H.; Zhang, Y.; Ma, D.; Xie, Y.; et al. Long-term nitrogen fertilizer improves nitrogen use efficiency and wheat yield by regulating the interactions of rhizosphere soil-root-microbes. *Agric. Ecosyst. Environ.* **2026**, *402*, 110317. [CrossRef]
60. Wang, X.; Gao, X.; Zhang, L.; Zhao, F.; Chen, J.; Zhang, W.; Chen, X. Optimal nitrogen fertilizer management increased soil organic carbon density by increasing particulate organic matter proportion and plant-derived carbon in topsoil. *Soil Tillage Res.* **2025**, *254*, 106764. [CrossRef]
61. Hoffland, E.; Kuyper, T.W.; Comans, R.N.J.; Creamer, R.E. Eco-functionality of organic matter in soils. *Plant Soil* **2020**, *455*, 1–22. [CrossRef]
62. de Vries, F.T.; van Groenigen, J.W.; Hoffland, E.; Bloem, J. Nitrogen losses from two grassland soils with different fungal biomass. *Soil Biol. Biochem.* **2011**, *43*, 997–1005. [CrossRef]
63. Zhou, Z.; Wang, C.; Zheng, M.; Jiang, L.; Luo, Y. Patterns and mechanisms of responses by soil microbial communities to nitrogen addition. *Soil Biol. Biochem.* **2017**, *115*, 433–441. [CrossRef]
64. Nadeem, F.; Mahmood, R.; Sabir, M.; Khan, W.U.; Haider, M.S.; Wang, R.; Zhong, Y.; Ishfaq, M.; Li, X. Foxtail millet [*Setaria italica* (L.) Beauv.] over-accumulates ammonium under low nitrogen supply. *Plant Physiol. Biochem. PPB* **2022**, *185*, 35–44. [CrossRef]
65. Rubio-Wilhelmi Mdel, M.; Sanchez-Rodriguez, E.; Rosales, M.A.; Blasco, B.; Rios, J.J.; Romero, L.; Blumwald, E.; Ruiz, J.M. Ammonium formation and assimilation in P(SARK)::IPT tobacco transgenic plants under low N. *J. Plant Physiol.* **2012**, *169*, 157–162. [CrossRef]
66. Rivero-Marcos, M.; Calleja-Satrustegui, A.; Ariz, I. The counteracting role of nitrate during ammonium toxicity in plants. *Hortic. Plant J.* **2026**, *12*, 49–61. [CrossRef]
67. Vandromme, C.; Spriet, C.; Putaux, J.L.; Dauvillée, D.; Courseaux, A.; D'Hulst, C.; Wattebled, F. Further insight into the involvement of PII1 in starch granule initiation in Arabidopsis leaf chloroplasts. *New Phytol.* **2023**, *239*, 132–145. [CrossRef]
68. Shi, N.; Yao, H.; Wang, G.; Zhao, G.; Wu, Y.; Zhang, G.; Kong, G.; Wang, N.; Bai, Y.; Du, Y.; et al. Topping influences crop photosynthesis and alters the absorption and redistribution of nutrients: A case study of tobacco. *Front. Plant Sci.* **2025**, *16*, 1597681. [CrossRef]
69. Chen, J.; Li, Y.; He, X.; Jiao, F.; Xu, M.; Hu, B.; Jin, Y.; Zou, C. Influences of different curing methods on chemical compositions in different types of tobaccos. *Ind. Crops Prod.* **2021**, *167*, 113534. [CrossRef]
70. Fu, Y.; Guo, H.; Cheng, Z.; Wang, R.; Li, G.; Huo, G.; Liu, W. NtNAC-R1, a novel NAC transcription factor gene in tobacco roots, responds to mechanical damage of shoot meristem. *Plant Physiol. Biochem.* **2013**, *69*, 74–81. [CrossRef]
71. Li, F.; Zhang, H.; Wang, S.; Xiao, W.; Ding, C.; Liu, W.; Guo, H. Identification of Topping Responsive Proteins in Tobacco Roots. *Front. Plant Sci.* **2016**, *7*, 582. [CrossRef]
72. Chen, X.; Sun, S.; Liu, F.; Shen, E.; Liu, L.; Ye, C.; Xiao, B.; Timko, M.P.; Zhu, Q.H.; Fan, L.; et al. A transcriptomic profile of topping responsive non-coding RNAs in tobacco roots (*Nicotiana tabacum*). *BMC Genom.* **2019**, *20*, 856. [CrossRef]
73. Zhang, X.; Pan, S.; Chen, D.; Pan, Y.; Bhanot, D.; Xu, T.; Gupta, S.; Zhang, J.; Huang, Y. WGCNA analysis and identification of key genes in tobacco in response to different nitrogen levels. *BMC Plant Biol.* **2025**, *25*, 465. [CrossRef]
74. Jiang, B.; Gao, L.; Wang, H.; Sun, Y.; Zhang, X.; Ke, H.; Liu, S.; Ma, P.; Liao, Q.; Wang, Y.; et al. Characterization and heterologous reconstitution of Taxus biosynthetic enzymes leading to baccatin III. *Science* **2024**, *383*, 622–629. [CrossRef]

75. Liu, B.; Song, Z.; Qi, X. Plant cytochrome P450 enzymes for bioactive metabolites biosynthesis, growth regulation, and stress adaptation. *Plant Physiol*. **2025**, 199. [CrossRef]
76. He, Y.; Zhang, K.; Li, S.; Lu, X.; Zhao, H.; Guan, C.; Huang, X.; Shi, Y.; Kang, Z.; Fan, Y.; et al. Multiomics analysis reveals the molecular mechanisms underlying virulence in Rhizoctonia and jasmonic acid-mediated resistance in Tartary buckwheat (*Fagopyrum tataricum*). *Plant Cell* **2023**, *35*, 2773–2798. [CrossRef]

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