

Article

Mechanism of Response of *Camellia sinensis* Cultivar ‘Ziyan’ Shoots Growth and Quality Metabolism to Nitrogen Levels

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Abstract

The shoots of ‘Ziyan’ (*Camellia sinensis* (L.) O. Kuntze) are rich in anthocyanins, making their flavor unique and providing health benefits. The problem is that ‘Ziyan’ has weak growth and low yield of the shoots. Nitrogen levels affect the yield and quality of tea shoots by regulating their growth and the metabolism of major quality components, but the underlying mechanism remains unclear. In this study, one-year-old seedlings of purple cultivar ‘Ziyan’ (*Camellia sinensis*) were grown hydroponically under three nitrogen levels (low nitrogen, moderate nitrogen, high nitrogen). The contents of quality-related chemical components and key enzyme activities were determined, combined with transcriptome analysis, to investigate the effects of nitrogen level on growth and quality component metabolism of ‘Ziyan’ tea seedlings. Results showed that compared with medium nitrogen (control), low nitrogen significantly decreased yield and total free amino acids, while high nitrogen did not markedly increase either index. Meanwhile, both low and high nitrogen significantly elevated phenylalanine content by 132.46% and 47.37%, respectively. Although the responses of EGCG and catechin contents to low nitrogen and high nitrogen are completely opposite. However, both low nitrogen and high nitrogen significantly reduced the anthocyanin content in shoots (8.15%, 25.26%), inhibiting anthocyanin synthesis. Kyoto Encyclopedia of Genes and Genome (KEGG) enrichment analysis revealed that flavonoid, phenylpropanoid and sucrose-related pathways were relatively active under both high and low nitrogen supply. Additionally, transcriptome analysis identified C4H, LAR and FLS as key genes, and transcription factors (e.g., GRF, bHLH, MYB) and auxins were actively involved in the nitrogen stress response related to ‘Ziyan’ shoot growth and major quality component metabolism. The findings help clarify the adaptive mechanisms of ‘Ziyan’ shoots under nitrogen stress and provide a scientific basis for the fertilization management.



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1. Introduction

The tea plant (*Camellia sinensis* (L.) O. Kuntze) is an important cash crop widely cultivated in many countries around the world. As a distinctive and competitive agricultural industry in China, tea plays a pivotal role in boosting the incomes of farmers in tea-growing regions and promoting the development of rural industries. Nitrogen metabolism directly determines the flavor, nutritional value and functional properties of tea by influencing the metabolism of quality components such as amino acids, tea polyphenols and caffeine [1,2].

Free amino acids, such as theanine and glutamic acid, form the core material basis of green tea quality, directly determining the fresh, crisp texture and aromatic profile of the tea liquor [3]. Tea polyphenols include secondary metabolites such as catechins, anthocyanins and flavonols [4], and their content levels and compositional ratios directly regulate the bitter and astringent flavor of tea [5]. The accumulation levels of catechins and flavonols are regulated by the enzymatic activities of cinnamic acid 4-hydroxylase (C4H), flavone synthase (FNS) and flavonol synthase (FLS); anthocyanins include components such as delphinidin, cyanidin and geraniidin, and their accumulation levels are regulated by the enzymatic activities of anthocyanidin synthase (ANS), leucoanthocyanidin reductase (LAR) and anthocyanidin reductase (ANR) [6]. Caffeine is an important bitter compound in tea leaves, possessing physiological effects such as stimulating alertness and alleviating fatigue. Appropriate nitrogen metabolism can enrich umami amino acids such as L-theanine and glutamic acid, thereby enhancing the freshness and vibrancy of the tea liquor; conversely, excessive nitrogen can impair the synthesis of quality components such as catechins, anthocyanins and caffeine, leading to a decline in tea quality and economic value [7]. In production, as nitrogen fertilizers can significantly promote tea plant growth and enhance tea yield and quality, the practice of excessive nitrogen application by tea farmers is prevalent [8]. This leads to ecological issues such as soil nutrient leaching and non-point source pollution, severely constraining the green and sustainable development of the tea industry [9].

The level of nitrogen supply influences the overall carbon-nitrogen metabolic regulation mechanism in tea plants, involving the synergistic action of various transcription factors, transport proteins and plant hormones, thereby affecting the growth and metabolic composition of tea plants [10]. Growth Regulating Factor (GRF) regulates cell division and elongation, integrates auxin and gibberellin signaling, and participates in bud and leaf growth [11–13]. The biosynthesis of quality components in young tea shoots, such as amino acids (e.g., theanine), flavonoids (e.g., catechins, anthocyanins) and caffeine, is also regulated by various transcription factors (e.g., MYB, bHLH). In tea plants, Cs**bHLH42-1** can directly bind to the promoters of Cs**DFR** and Cs**ANS**, key genes in the anthocyanin and catechin biosynthesis pathways, whilst also enhancing the transcriptional activation capacity of Cs**MYB75** on target genes, thereby synergistically promoting the accumulation of anthocyanins and catechins in young shoots [14,15]. Comparative analysis of young leaves from wild and cultivated tea plants suggests that 15 MYB and bHLH transcription factors may be involved in the coordinated regulation of flavonoid and amino acid biosynthesis [16]. Nitrogen metabolism is closely linked to the primary and secondary metabolic networks of tea plants, serving as a central hub connecting carbon metabolism with the formation of tea leaf quality; elucidating the patterns of nitrogen metabolic responses in tea plants under different nitrogen levels is a crucial theoretical prerequisite for achieving precision fertilization in tea plants and ensuring both high yields and superior quality.

‘Ziyan’ is a distinctive and high-quality purple-bud tea variety. Its young shoots can accumulate up to 3% anthocyanins, endowing the leaves with a unique color, distinctive flavor characteristics and excellent health benefits; it enjoys broad market prospects and high consumer recognition [17,18]. Previous field observations by our research group have revealed that, under standard cultivation conditions, compared to conventional green tea varieties, ‘Ziyan’ tea plants exhibit slower growth rates, weaker vegetative vigor, and lower overall yield levels. The team has previously conducted systematic research into the regulatory mechanisms by which light influences the yield and quality development of ‘Ziyan’ new shoots, as well as anthocyanin biosynthesis [19–21]. Our research has revealed that high nitrogen supply increases the levels of arginine, asparagine and glutamine in the root system, whilst simultaneously reducing the levels of sucrose, polyphenols and

caffeine. We have also found that transcription factors (such as AP2/ERF, MYB and WRKY) and auxins are actively involved in the response of ‘Ziyan’ roots to high nitrogen stress [10]. However, to date, the regulatory mechanisms of nitrogen metabolism in the new shoots of ‘Ziyan’ under different nitrogen supply conditions, as well as the molecular regulatory networks mediating the accumulation of quality-related metabolites and the growth and development of new shoots, remain unclear.

2. Materials and Methods

2.1. Plant Materials and Treatment

One-year-old cuttings of the purple tea cultivar ‘Ziyan’ were obtained from Sichuan Muchuan Yizhichun Tea Co., Ltd. (Leshan, China). All plant materials were pre-cultivated in the No.5 Education Greenhouse at the Chengdu Campus of Sichuan Agricultural University (Chengdu, China). After acclimation in deionized water for 7 days, seedlings were cultured with 1/4-strength Xiaoxi Maoyi nutrient solution (containing $1.427 \text{ mmol} \cdot \text{L}^{-1} \text{ N}$, $\text{NH}_4^+ \text{-N}:\text{NO}_3^- \text{-N} = 3:1$, pH 5.0) for another 7 days, and then transferred to 1/2-strength tea-specific nutrient solution. The nutrient solution was renewed every 7 days until new adventitious roots emerged. Once the root system was well-developed, uniform and healthy tea seedlings with consistent growth status were selected for nitrogen (N) treatments. Three N levels were applied: low nitrogen ($0.3 \text{ mmol} \cdot \text{L}^{-1}$, LN), medium nitrogen ($1.5 \text{ mmol} \cdot \text{L}^{-1}$, MN), and high nitrogen ($4.5 \text{ mmol} \cdot \text{L}^{-1}$, HN) [22]. Each treatment contained 10 pots, with 6 seedlings per pot. Plants were hydroponically grown in 6 L of nutrient solution, which was replaced every 7 days. Continuous aeration was provided by an air pump for 24 h per day. The nutrient solution consisted of macronutrients (P 0.1, K 1.0, Ca 0.8, Mg $0.4 \text{ mmol} \cdot \text{L}^{-1}$) and micronutrients (EDTA-Fe 6.3, Mn 1.5, Zn 1.0, Cu 0.2, B 10, Mo 0.5, and Mo $50 \mu\text{mol} \cdot \text{L}^{-1}$). All experiments were performed under controlled conditions: light/dark cycle of 14 h/10 h, temperature of $25 \pm 1 \text{ }^\circ\text{C}$ (day)/ $20 \pm 1 \text{ }^\circ\text{C}$ (night), 70% relative humidity, and light intensity of $200 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ [20].

2.2. Analysis of Shoot Yield and Biochemical Indicators

2.2.1. Measurement of Phenotypic Characteristics of Tea Seedlings

Sampling was conducted when the majority of young shoots reached the one-bud-two-leaves (1B2L) stage. The length and fresh weight of the harvested samples (1B2L) were measured, and the fresh yield of buds and leaves per plant was recorded. All young shoot samples were collected in batches, thoroughly mixed to eliminate individual differences, and equally divided into three independent subsamples for subsequent analyses of physiological characteristics, quality-related components, and transcriptome sequencing, respectively.

2.2.2. Determination of Free Amino Acids and Enzyme Activity

The content of total free amino acids was determined according to the Chinese National Standard (GB/T 8314-2013) [23]. Briefly, 1.0 g of milled freeze-dried sample was mixed with 150 mL of boiling water, and then extracted in a $100 \text{ }^\circ\text{C}$ water bath for 45 min. After extraction, the absorbance of the supernatant was measured at 570 nm in the visible region via the ninhydrin colorimetric method, and the total amino acid content in the extract was calculated based on the standard calibration curve. For profiling of individual amino acid components, high-performance liquid chromatography (HPLC) was applied following the procedures specified in GB/T 30987-2020 [24].

At the same time, the activities of glutamine synthetase (GS), glutamate dehydrogenase (GDH), and glutamate synthase (GOGAT) in fresh shoots from each treatment were determined using commercial colorimetric assay kits purchased from Nanjing Jiancheng

Technology Co., Ltd. (Nanjing, China), in strict accordance with the manufacturer's instructions [10]. Specifically, GS activity was quantified by detecting absorbance at 540 nm, GDH activity was measured via absorbance changes at 340 nm, and GOGAT activity was quantified at 506 nm. All measurements were performed with three biological replicates for each treatment.

2.2.3. Determination of Tea Polyphenol, Catechin, and Caffeine Contents

Tea polyphenols were detected according to the National Standard of China (GB/T 8313-2018) [25]. The extraction method was as follows: A total of 0.5 g of crushed freeze-dried sample was placed in a 10 mL centrifuge tube, and then 5 mL of 70% methanol preheated at 70 °C was added. After extraction for 30 min in a 70 °C water bath, the extracted solution was centrifuged to obtain the supernatant. Again, the extraction was repeated with 5 mL of 70% methanol. According to the Folin–Ciocalteu method, tea polyphenols were measured at 765 nm using a UV spectrophotometer (UV2300, Jinghua Technology Instrument, Shanghai, China). Meanwhile, the extracts were also prepared to determine the catechin components and caffeine contents using high-performance liquid chromatography (Agilent 1260, Agilent Technologies, Palo Alto, CA, USA). Subsequently, the test parameters were set according to the National Standard.

2.2.4. Quantification of Anthocyanins

Anthocyanins were extracted from liquid nitrogen-frozen leaf powder using acidified methanol, hydrolyzed, and quantified by HPLC against external standards [19]. Briefly, 0.2 g powder was extracted with 4 mL of 1% HCl in methanol (*v/v*) at 4 °C for 2 h, centrifuged (5000 rpm, 10 min), and re-extracted twice (2 h and 12 h). The pooled extracts were hydrolyzed (0.4 mL 5 mol/L HCl, 90 °C, 30 min), cooled on ice, and analyzed by HPLC (Agilent 1260, VWD detector, Titank C18 column, 5 µm) using a gradient of 15–30% B (acetonitrile) over 30 min [mobile phase A: water-acetonitrile-formic acid (87:3:10, *v/v/v*)]. Operating conditions: 1.0 mL/min, 10 µL injection, 35 °C column temperature, 520 nm detection. Quantification was performed using peonidin, pelargonidin, delphinidin, malvidin, and cyanidin chlorides (ChromaDex, Los Angeles, CA, USA) as external standards.

2.3. Transcriptome Analysis

For transcriptome profiling, total RNA was extracted from shoot samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the supplier's protocol. RNA quality was assessed by spectrophotometry (NanoDrop ND-1000) and capillary electrophoresis (Agilent 2100 Bioanalyzer Agilent Technologies, Santa Clara, CA, USA) to verify concentration, purity (A260/A280 ratio), and integrity (RNA integrity number, RIN). Messenger RNA was enriched from 5 µg of total RNA by two successive rounds of oligo(dT) magnetic bead capture. The purified mRNA was chemically fragmented under heat in the presence of divalent metal ions, and strand-specific cDNA libraries were prepared using the Illumina mRNA-Seq Sample Preparation Kit. Sequencing was carried out commercially on an Illumina HiSeq 4000 instrument (150 bp paired-end mode; Hangzhou Lianchuan Biotechnology, Hangzhou, China), with each treatment represented by three independent biological replicates.

Bioinformatic processing followed a standard pipeline: raw reads were trimmed with Cutadapt to eliminate adapter contamination, low-quality bases, and ambiguous sequences (>5% N). High-quality reads were aligned to the 'Suchazao' tea genome (*Camellia sinensis*) with HISAT2 [26], and alignment quality was inspected with FastQC (v0.11.6). Assembled transcripts were functionally characterized by homology searches against publicly available databases. To uncover enriched biological pathways, KEGG enrichment was conducted with a significance cutoff of $E \leq 10^{-5}$. Gene abundance was expressed as normalized

read counts, and DEGs were called when $|\log_2 \text{fold change}| \geq 1$ and false discovery rate $q < 0.05$.

2.4. Quantitative Real-Time PCR (qRT-PCR) Analysis

To independently verify the RNA-seq results, ten DEGs were chosen at random for qRT-PCR assays. First-strand cDNA was synthesized from total RNA with the PrimeScript™ RT Reagent Kit (gDNA Eraser Plus; Takara, Kyoto, Japan). Locus-specific primer pairs were computationally designed against the NCBI reference sequences and are provided in Table S1. Amplification reactions (20 µL) contained SYBR® Premix Ex Taq™ II (Takara) and were run on a real-time thermal cycler under the cycling parameters reported by Li et al. [20]. The glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) served as the endogenous control. Each sample was assayed in triplicate (three biological × three technical replicates), and fold changes in transcript abundance were computed by the comparative 2^{-DDct} method.

2.5. Statistical Analysis

All phenotypic, biochemical, and gene expression data were analyzed with SPSS 20.0 (IBM Corp., Chicago, IL, USA). Treatment means were compared by one-way analysis of variance (ANOVA), and pairwise differences were separated by the least significant difference (LSD) post hoc test at a significance level of $\alpha = 0.05$. Data are presented as mean ± standard deviation (SD) unless otherwise stated.

3. Results

3.1. Yield, Quality Components and Enzymes of Shoots

As shown in Figure 1A–C, shoot length, 100-shoot fresh weight, and fresh yield all displayed a “rise-fall” pattern with increasing nitrogen supply. Meanwhile, tea polyphenol contents in shoots gradually decreased, whereas amino acid and caffeine contents showed an increasing trend. Compared with the MN group, 100-shoot weight and yield in the HN group were reduced by 23.18% and 3.70%, respectively, while both parameters were significantly lower in the LN group ($p < 0.05$). Tea polyphenol content was significantly decreased by 20.81% in the HN group, whereas caffeine content was significantly increased by 22.33%. No significant difference in amino acid content was observed between the HN and MN groups ($p < 0.05$). Collectively, relative to the MN treatment, the HN condition did not significantly improve shoot yield or amino acid accumulation, but markedly reduced tea polyphenol content. Consistent with these findings, the activities of key nitrogen metabolic enzymes including GS, GOGAT, and GDH in tea shoots also exhibited corresponding changes (Figure 1D).

3.2. Analysis of Differentially Expressed Genes

RNA-seq was conducted to decipher the transcriptional architecture governing nitrogen-modulated shoot growth in ‘Ziyan’. cDNA libraries from nine samples (three nitrogen levels × three biological replicates) were sequenced. Differential expression analysis identified 403 DEGs in the LN group (ZML vs. ZLL; 65 upregulated, 338 downregulated) and 234 DEGs in the HN group (ZML vs. ZHL; 53 upregulated, 181 downregulated) (Figure 2A–C). Notably, the LN comparison yielded more DEGs and a higher proportion of downregulated genes than the HN comparison.

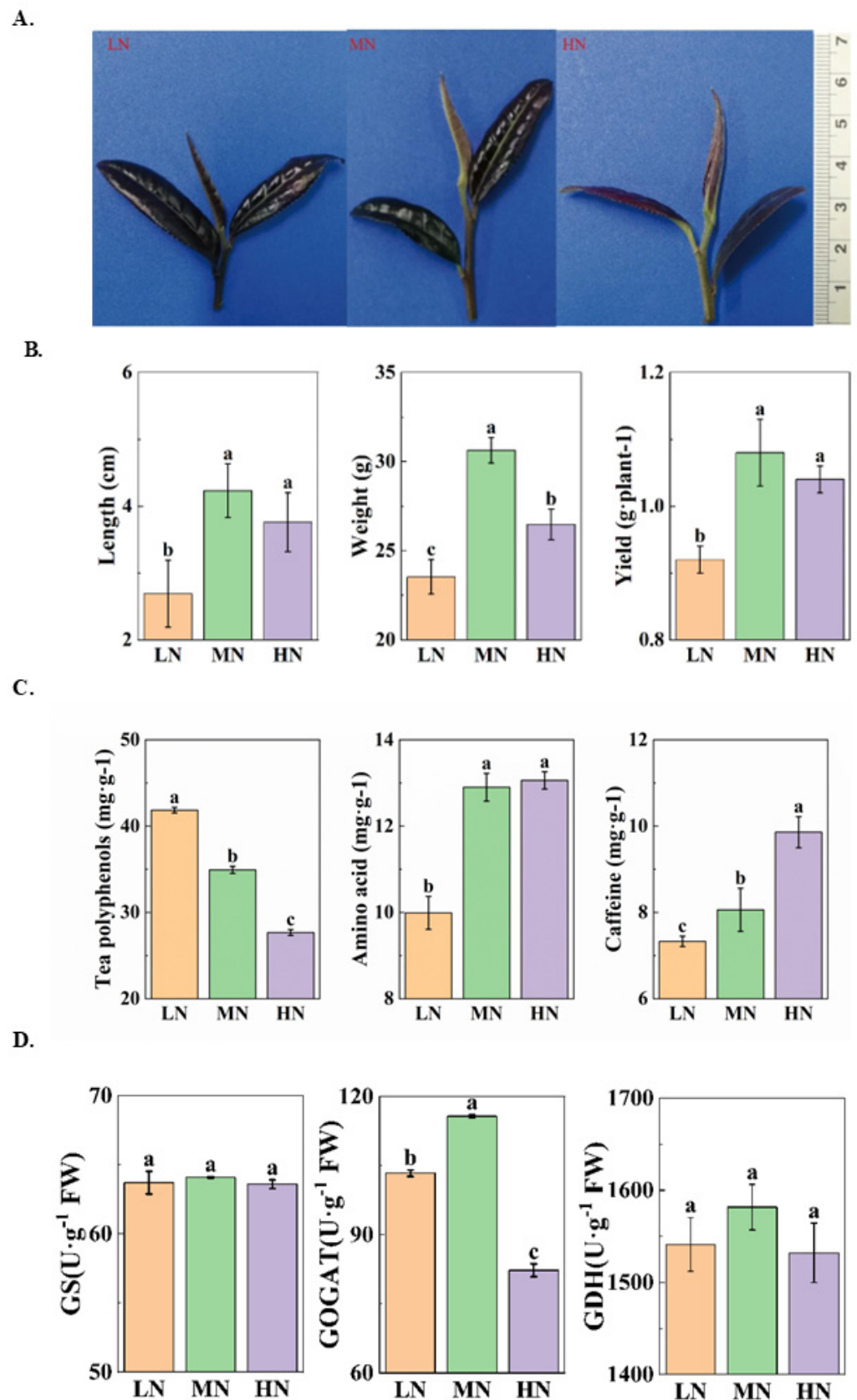


Figure 1. Shoot growth and related physiological indicators under different nitrogen treatments (LN: low nitrogen; MN: medium nitrogen; HN: high nitrogen). (A) Shoot growth performance; (B) Length, 100-fresh-shoot weight and yield of fresh shoots; (C) Quality-related chemical components; (D) Activities of nitrogen metabolic enzymes. Different letters indicate significant differences at $p < 0.05$.

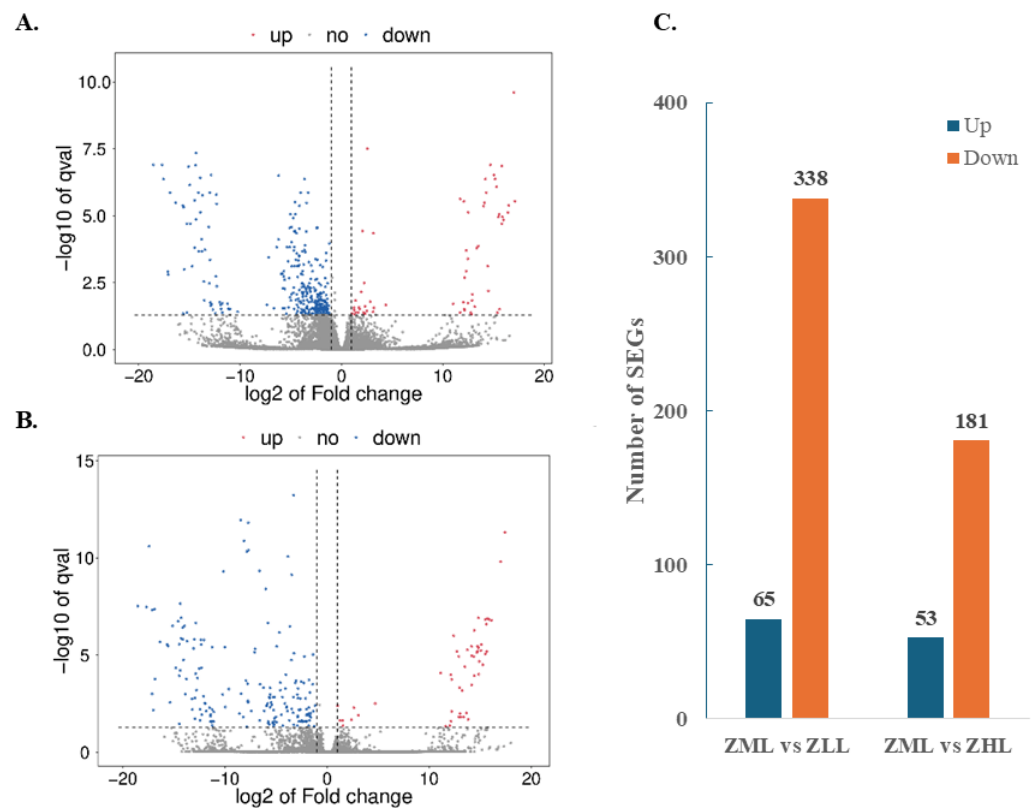


Figure 2. Analysis of differentially expressed genes (DEGs) in shoots under different nitrogen treatments (LN: low nitrogen; MN: medium nitrogen; HN: high nitrogen). (A,B) Volcano plots showing DEGs in the comparison groups of ZML vs. ZLL and ZML vs. ZHL, respectively; (C) Statistical analysis of DEG quantities. DEGs were defined as genes with $|\log_2(\text{fold change})| \geq 1$ and adjusted $p < 0.05$.

3.3. GO and KEGG Enrichment Analysis

GO analysis was performed for functional annotation of the identified DEGs, with results shown in Figure 3. GO classification divided DEGs into three main categories: biological processes, cellular components, and molecular functions. The DEGs were mainly enriched in terms including transcription regulation, oxidation–reduction processes, cytoplasm, DNA binding and transcription factor activity, as well as protein binding (Figure 3A,B). The comparative profiles between the LN subgroups exhibited high similarity.

At the same time, KEGG pathway analysis was subsequently performed for the identified DEGs, with results included in Figure 3. KEGG enrichment analysis indicated that DEGs in the LN group were predominantly associated with pathways including plant hormone signal transduction, plant MAPK signaling pathway, glycolysis/gluconeogenesis, galactose metabolism, cysteine and methionine metabolism, pentose phosphate pathway, and flavonoid biosynthesis (Figure 3C). In contrast, DEGs in the HN group were mainly enriched in amino sugar and nucleotide sugar metabolism, starch and sucrose metabolism, phenylpropanoid biosynthesis, galactose metabolism, ABC transporters, and nitrogen metabolism (Figure 3D). Collectively, DEGs in shoots of the ‘Ziyan’ cultivar were mainly concentrated in pathways such as flavonoid biosynthesis, phenylpropanoid biosynthesis, starch and sucrose metabolism, galactose metabolism, and plant hormone signal transduction.

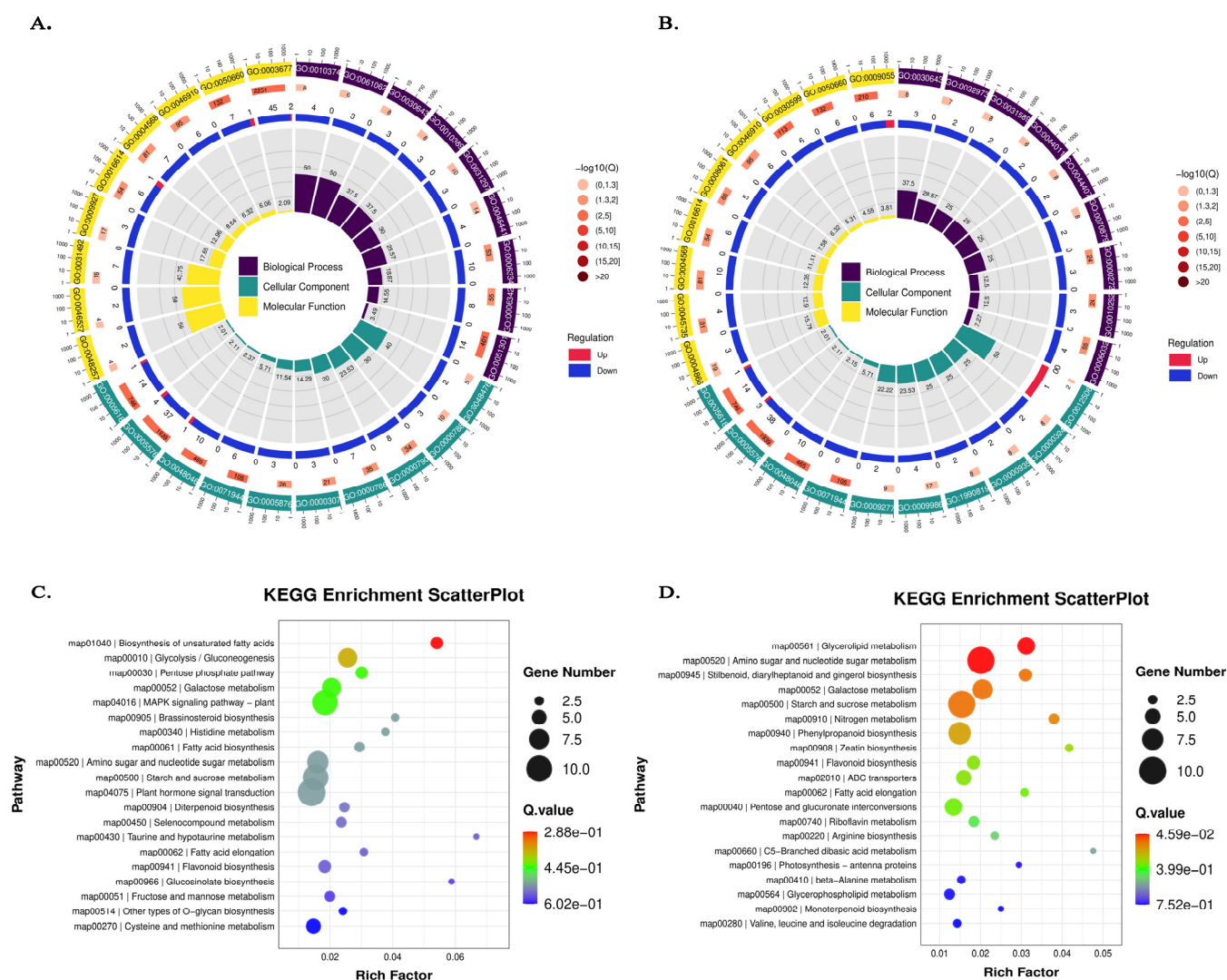


Figure 3. GO and KEGG enrichment analyses of differentially expressed genes (DEGs) in shoots under different nitrogen treatments. (A,B) GO enrichment for ZML vs. ZLL and ZML vs. ZHL, respectively; (C,D) KEGG enrichment for ZML vs. ZLL and ZML vs. ZHL, respectively. LN: low nitrogen; MN: medium nitrogen; HN: high nitrogen.

3.4. Analysis of Amino Acids and Differentially Expressed Genes

Amino acid metabolism mainly involves five major amino acid families, namely the glutamate family, aspartate family, alanine family, serine family, and aromatic amino acid family, which are distinguished by different colors in the metabolic map (Figure 4; Table S2). In the present study, elevated nitrogen supply was found to induce an increasing trend in the contents of amino acids belonging to the glutamate family, aspartate family, and alanine family. By contrast, the serine family displayed an initial increase followed by a decrease, whereas the aromatic amino acid family showed the opposite pattern. Relative to the MN group, the LN and HN groups exhibited significantly higher contents of tyrosine, phenylalanine, isoleucine, and leucine in shoots. Specifically, phenylalanine levels were increased by 132.46% and 47.37%, respectively ($p < 0.05$). Moreover, the HN group showed markedly higher contents of theanine, glutamate, asparagine, and alanine, among which asparagine and alanine were elevated by 74.50% and 46.34%, respectively. No significant differences were observed in the contents of glutamine, aspartate, and arginine ($p < 0.05$). As illustrated in Table S5, the LN group showed downregulated expression of asparagine synthase (ASN), serine decarboxylase (SDC), and dihydroxyacid dehydrogenase (DHAD),

together with upregulated expression of tryptophan synthase (TrpB2). In the HN group, phosphoenolpyruvate carboxykinase (PCK), raffinose synthase (RAFS2), and α -amylase (AMY) were upregulated, whereas sucrose synthase (SuSy1) was downregulated.

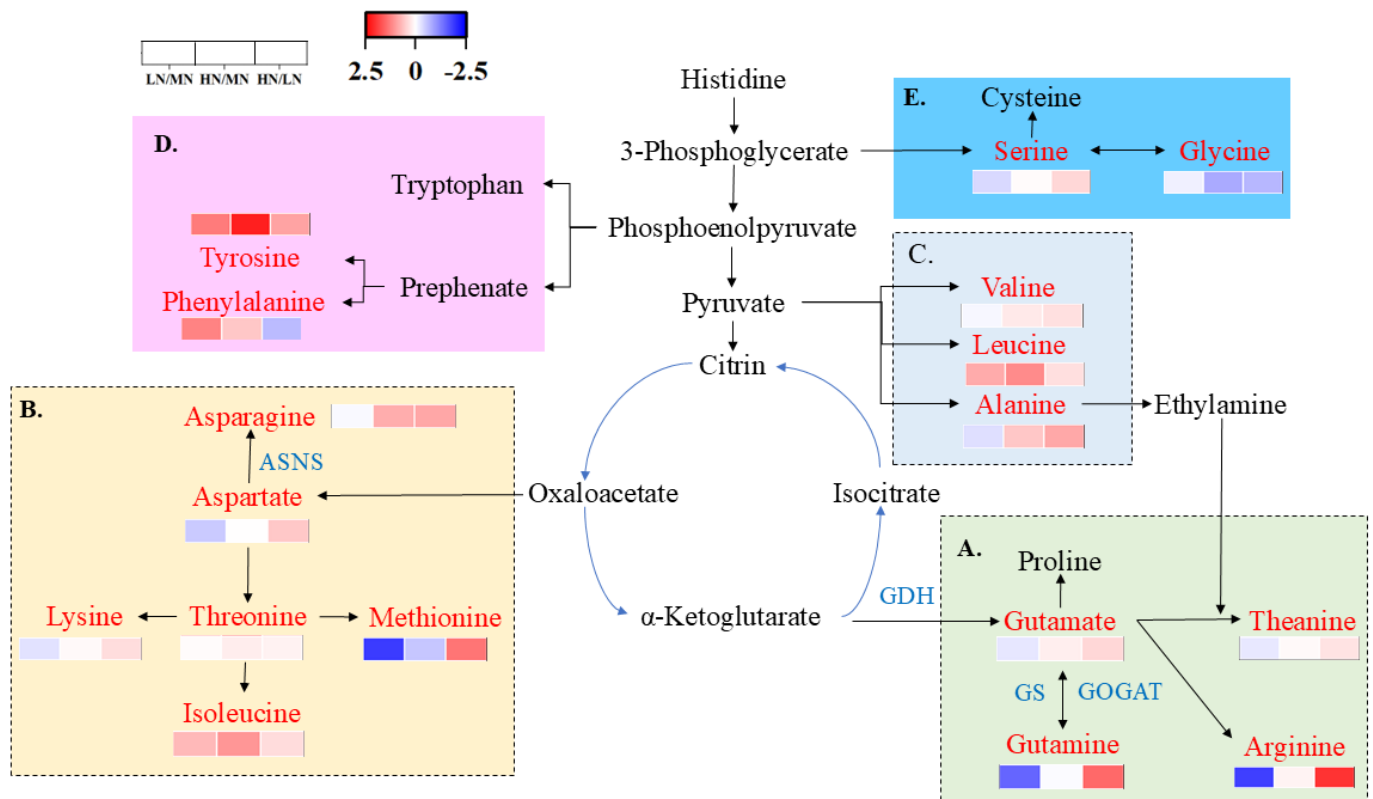


Figure 4. Variations in free amino acids of shoots under different nitrogen conditions. (A) Glutamate family (light-green frame); (B) Aspartate family (yellow frame); (C) Alanine family (light gray frame); (D) Serine family (blue frame); (E) Aromatic amino acids family (purple frame). A color gradient from red (high abundance) to blue (low abundance) is employed to represent the relative abundance of metabolites. LN: low nitrogen; MN: medium nitrogen; HN: high nitrogen.

Taken together, these findings demonstrate that increasing nitrogen levels promote the accumulation of dominant amino acids in shoots. Low-nitrogen conditions suppressed the biosynthesis of major amino acids, whereas high-nitrogen conditions enhanced synthesis of amino acids such as theanine, glutamic acid, asparagine, and alanine. In addition, high nitrogen affected sucrose and starch metabolism via the tricarboxylic acid cycle.

3.5. Analysis of Catechins and Anthocyanins and Differential Gene Expression

As shown in Figure 5 and Table S3, compared with those in the MN group, the EGCG and ECG contents of shoots in the LN group were significantly higher by 33.55% and 31.75%; whereas the EGC and EC contents were significantly lower by 32.03% and 25.15%, respectively ($p < 0.05$). The EGCG, EGC, and EC contents in the HN group were significantly lower by 23.24%, 34.47%, and 17.18%, respectively ($p < 0.05$). These results indicate that the EGC and EC contents were reduced significantly in the LN group and the HN group, while EGCG content showed opposite trends in these two groups.

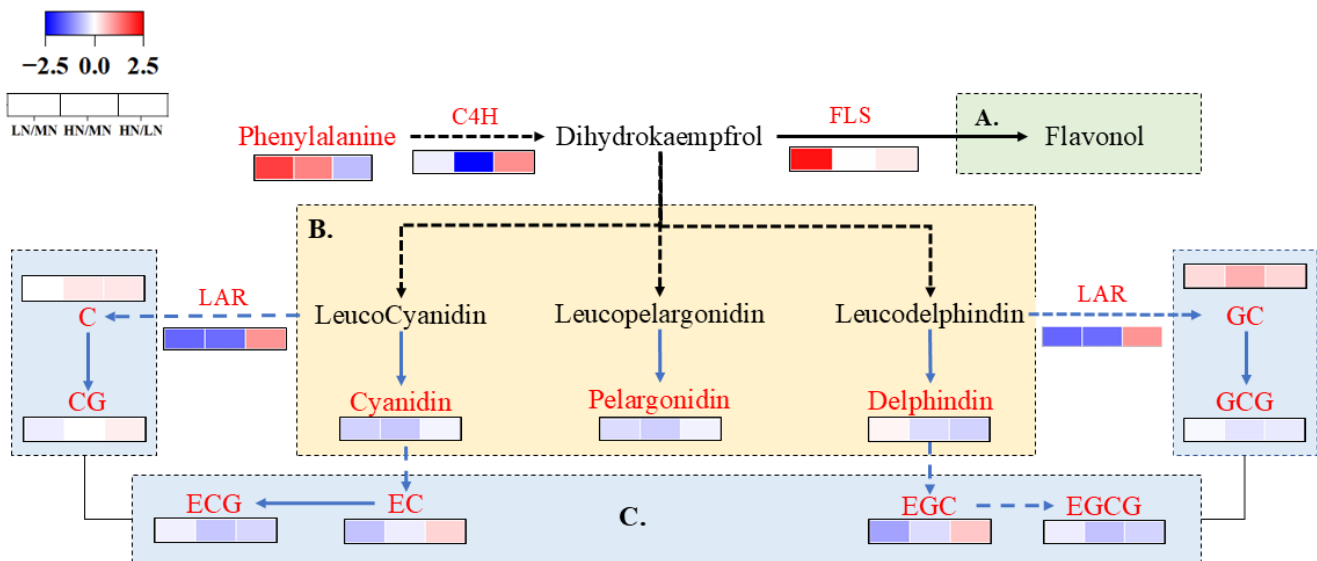


Figure 5. Variations in tea polyphenols of shoots under different nitrogen conditions. (A): Flavonol pathway (light green box); (B): Anthocyanin pathway (yellow box); (C): Catechin pathway (light blue box). LN: low nitrogen; MN: medium nitrogen; HN: high nitrogen.

Meanwhile (Figure 5; Table S4), compared with those in the MN group, only the delphinidin content in the LN group was significantly higher (7.57%) among the anthocyanin components, while the total anthocyanin content was 8.15% lower. The contents of delphinidin, cyanidin, pelargonidin and total anthocyanin in the HN group were lowest, which were lower by 20.66%, 30.68%, 27.61%, and 25.26%, respectively ($p < 0.05$).

Furthermore, the phenylalanine contents of shoots were both significantly increased under low nitrogen and high nitrogen; however, Cinnamate 4-Hydroxylase (C4H) and LAR (Leucoanthocyanidin Reductase) were downregulated, while FLS (Flavonol Synthase) was upregulated, resulting in reduced flux toward anthocyanin and catechin synthesis. These results indicated that the contents of most catechins and anthocyanins in shoots were both reduced under low nitrogen and high nitrogen.

3.6. Analysis of the Differential Expression Profiles of Key Genes

It revealed that genes encoding transcription factors, growth factors, and transport proteins associated with shoot growth were differentially expressed under different nitrogen conditions. For transcription factors (TFs) (Figure 6A; Table S5), a total of 29 DEGs were identified. These differentially expressed genes were primarily concentrated in 6 GRF, 4 bHLH, 3 F-box, 2 MYB, 2 BZIP, and 1 WRKY families involved in synthesis and signal transduction. With the exception of FAMA-X1, these DEGs in the LN group and the HN group were both downregulated. At the same time, we identified 14 growth-related DEGs (Figure 6B; Table S5), including 6 auxin-related genes and 6 cell cycle-related genes; these DEGs in the LN group and the HN group were both downregulated. Regarding transporters (Figure 6B; Table S5), a total of 18 DEGs were identified, including 9 nutrient transporters, 4 ABC transporters, 2 nitrogen/amino acid transporters, and a small number of lipid and organic acid transporters. Except for ABC-I17, which was upregulated in the HN group, the remaining differentially expressed genes were downregulated. Among them, nitrate transporter 2.5 (NRT2.5), amino acid permease (AAP), and aquaporin (AQP6) were downregulated in the HN group, while phosphate transporter (PHT1) and aquaporin (PIP2-1) were downregulated in the LN group.

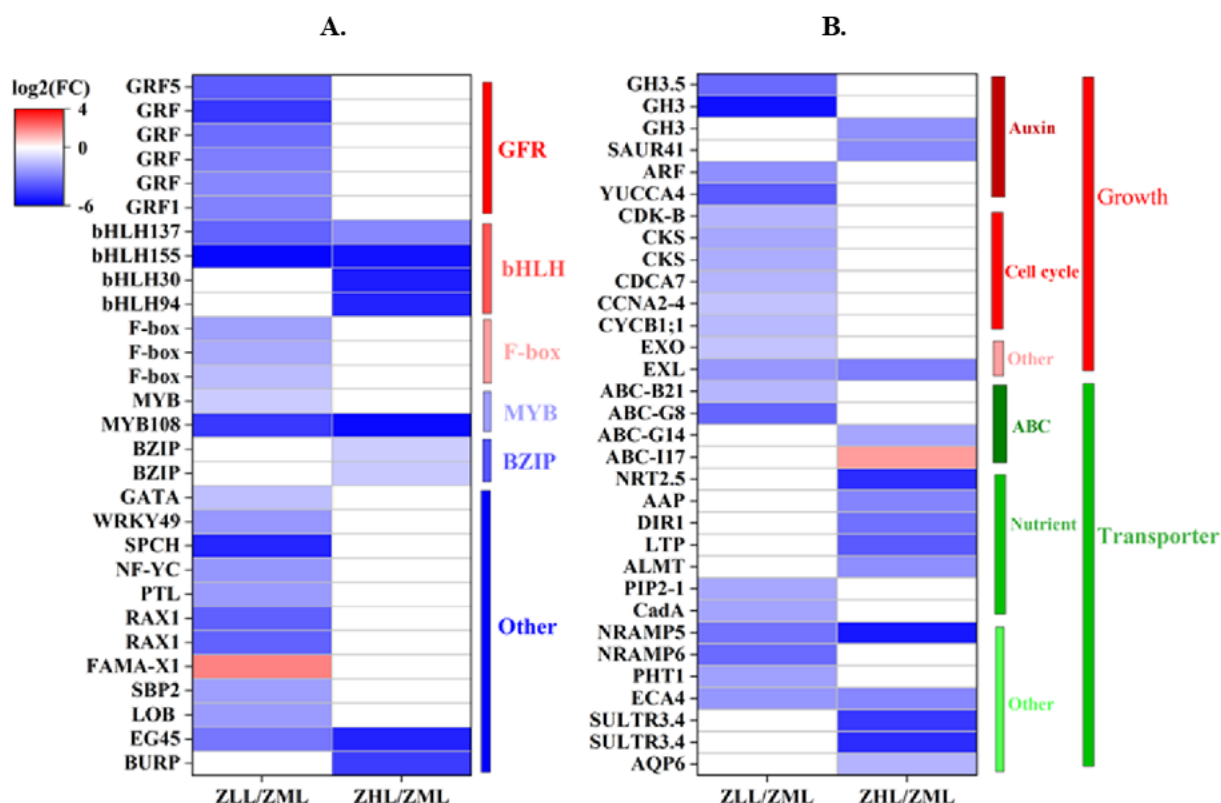


Figure 6. Expression patterns of related genes in shoots under nitrogen treatments. (A): transcription factor. (B): growth and transporter. DEGs are represented by different colors ranging from low (blue) to high (red); the same below. LN: low nitrogen; MN: medium nitrogen; HN: high nitrogen.

3.7. qRT-PCR Validation of Gene Expression

Quantitative real-time PCR (qRT-PCR) analysis of the expression profiles of ten selected genes revealed a high degree of consistency with RNA-seq data, which effectively validated the reproducibility and reliability of our transcriptomic results (Figure S1). It included 2 transcription factors (bHLH155 and BZIP), 3 amino acid transporters (ABC-G14, AQP6, ABC-I17), 2 sucrose metabolism genes (SuSy1, PCK), and 3 regulators of growth and resistance (RAFS2, MT protein, NIMIN2).

4. Discussion

4.1. Mechanisms Underlying the Effects on the Growth for ‘Ziyan’ Shoots in Different Nitrogen Levels

The growth of young shoots was a key factor affecting tea yield, and it was influenced by nitrogen supply [27,28]. Auxin synthesis and transport, as well as cell cycle-related genes, were involved in regulating the growth of the shoots [29–31]. Auxin synthesis and transport were also influenced by transcription factors such as F-box, bHLH, and MYB [32–34]. The F-box protein TIR1/AFB functions as an auxin receptor that mediates the ubiquitination and degradation of AUX/IAA repressors, thereby positively regulating the activity of ARF transcription factors and triggering the expression of auxin-responsive genes [35]. In Arabidopsis, downregulated expression of MYB73/MYB77 in shoots directly inhibited the transcription of auxin-responsive genes [36,37]. In Apple, MdbHLH3 specifically bound to their promoters to inhibit MdpIN1 and activate MdGH3-2, respectively, thereby reducing free auxin levels in leaves [38].

In this study, length, weight, and yield of the shoots were significantly reduced under low nitrogen. This may be due to the downregulation of F-box, bHLH, and MYB genes,

which, through the significant downregulation of key cell cycle genes (CDK-B, CCNA2-4, CYCB1;1), combined with the inhibition of auxin synthesis and signaling (YUCCA4, ARF) and ABC transporters (ABC-B21, ABC-G8), leading to cell arrest and growth inhibition in the shoots (Figure 6). Meanwhile, length and weight of the shoots were significantly reduced under high nitrogen, likely due to downregulated expression of bHLH, MYB, and BZIP genes, as well as inhibition of hormone signaling and cell expansion (SAUR41, EXL, GH3) were suppressed, reducing substance transport (AAP, ABC-G14, NRAMP5, SULTR3.4, ALMT, AQP6) (Figure 6; Table S5), and inhibiting sucrose synthesis (RAFS2 upregulated, SuSy1 downregulated), thereby leading to impaired shoot growth. These results indicated that the growth of the shoots was inhibited in low nitrogen and high nitrogen. This phenomenon may be attributed to the involvement of transcription factors in the expression of genes associated with the cell cycle and auxin, which simultaneously affect the carbon and nitrogen metabolism of tea plants [39–41]. The specific mechanisms underlying these effects require further research to confirm.

4.2. Mechanisms Underlying the Effects of Amino Acids and Secondary Metabolites for ‘Ziyan’ Shoots in Different Nitrogen Levels

Amino acid metabolism not only served as a central component in the nitrogen metabolism of tea plants but also exerted a remarkable impact on tea flavor [42,43]. Under low nitrogen, the contents of arginine, glutamine, theanine, aspartic acid, serine, and methionine in shoots were significantly reduced, while the expression of aspartate synthase (ASNS), serine decarboxylase (SDC), and dihydroxyacid dehydrogenase (DHAD) was also downregulated (Table S5). This further demonstrated that low nitrogen supply inhibited the synthesis of major amino acids in tea shoots [44,45]. High nitrogen selectively increased the accumulation of specific functional amino acids in tea seedlings without improving the overall total amino acid pool. Tea shoots preferentially accumulate nitrogen-responsive amino acids (e.g., asparagine and theanine) to adapt to excess nitrogen conditions [46]. Metabolite analyses showed that high nitrogen significantly elevated the levels of glutamic- and aspartic acid-derived amino acids, including theanine, glutamic acid, asparagine, and alanine, relative to medium-nitrogen conditions (Figure 4; Table S2), whereas the contents of glutamine and arginine remained unchanged. Transcriptomic data indicated that the upregulation of amino acid biosynthetic genes redirects carbon skeletons toward amino acid production, thereby promoting the enrichment of functional amino acids under high nitrogen. Overall, excess nitrogen remodels tea amino acid metabolism by altering amino acid composition rather than universally enhancing total amino acid synthesis.

Meanwhile, phenylalanine acted as a key precursor in tea polyphenol biosynthesis. It was converted into flavonoids and flavonoid glycosides through naringenin, after which anthocyanins were generated, and catechins were ultimately synthesized [2] (Figure 5). As typical carbon-derived secondary metabolites, flavonoid biosynthetic pathways were considerably regulated by nitrogen availability [47,48]. Interestingly, although the phenylalanine content in shoots increased significantly under high nitrogen and low nitrogen, the contents of most anthocyanin and catechin components decreased (compared with the medium nitrogen). This may be due to reduced C4H and LAR activity, coupled with increased FLS activity, which reduced the flux toward anthocyanin and catechin synthesis. The reduction in catechin and anthocyanin synthesis also corroborated the finding that the increase in phenylalanine was greater than that of the glutamic acid and aspartic acid families. Generally, nitrogen deficiency notably upregulated the expression levels of enzymes associated with anthocyanin biosynthesis, including PAL, F3H, ANS, and DFR, in plant foliage, which in turn modulates anthocyanin accumulation [49]. In purple waxy maize, a reasonable nitrogen application rate enhanced the activity of enzymes involved in anthocyanin biosynthesis (PAL, F3H, and DFR) and, by providing a relatively abundant car-

bon skeleton, significantly promoted anthocyanin accumulation [50]. Under high-nitrogen supply conditions, the caffeine content in shoots was elevated, which competed with amino acid biosynthesis for carbon and nitrogen resources and thus led to no significant variation in free amino acid contents when compared with moderate nitrogen levels.

Furthermore, most transcription factors of MYB, bHLH, and WRKY positively regulated the biosynthesis of catechins and anthocyanins. For instance, CsMYB5b enhanced the biosynthesis of catechins and proanthocyanidins in tobacco leaves by upregulating the expression of LAR and ANR [51,52], while overexpression of PpMYB108 significantly increased anthocyanin content [53]. VabHLH137 in grapes promoted the biosynthesis of proanthocyanidins and anthocyanins [54], whereas increased MdWRKY1 expression also facilitated the transcription of anthocyanin-related genes and anthocyanin accumulation [55]. In this study, we observed that the expression levels of bHLH, MYB, and WRKY genes in shoots were downregulated under both low-nitrogen and high-nitrogen conditions (Figure 6; Table S5), suggesting that the synthesis of major catechins and anthocyanins were inhibited. This may be because the synthesis and accumulation of carbohydrates and flavonoids were inhibited under low nitrogen [56], whereas more carbon skeletons were allocated to amino acid synthesis under high nitrogen [57,58].

5. Conclusions

In this study, we analyzed transcriptomic profiles related to amino acid, catechin and anthocyanin biosynthesis in ‘Ziyan’ tea shoots under varying nitrogen levels. Nitrogen deficiency reduced shoot yield and quality, while excess nitrogen did not increase yield or total free amino acids but markedly lowered anthocyanin and polyphenol contents, impairing tea processing quality. Phenylalanine biosynthesis was transcriptionally induced under both low and high nitrogen, whereas the anthocyanin pathway was strongly suppressed. This physiological response is modulated by the coordinated metabolic network, including carbon–nitrogen crosstalk and the combined effects of transcription factors, transporters and phytohormones, and the underlying mechanisms remain to be further explored. Since the results were obtained from controlled nutrient solution culture, field trials in commercial tea plantations are needed for validation. This study advances our knowledge of nitrogen regulation in tea and provides a theoretical basis for rational nitrogen management of this purple tea cultivar.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/horticulturae12070804/s1>, Figure S1: RT-qPCR validation of some genes under nitrogen treatment; Table S1: primers used for qRT-PCR analysis; Table S2: amino acid component content of shoots under different nitrogen treatments; Table S3: catechin components of shoots under different nitrogen treatments. Table S4: anthocyanidin contents of shoots under different treatments. Table S5: expression patterns of related genes under different nitrogen treatments. Table S6: The key gene expression level information.

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Abbreviations

The following abbreviations are used in this manuscript:

GC	Galocatechin
EGC	Epigallocatechin
C	Catechin
EC	Epicatechin
EGCG	Epigallocatechin Gallate
GCG	Galocatechin Gallate
ECG	Epicatechin Gallate
CG	Catechin Gallate

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