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Research article

Effects of nitrogen supply on flavonol glycoside biosynthesis and accumulation in tea leaves (Camellia sinensis)

Fang Dong[a], Jianhui Hu[b], Yuanzhi Shi[a], Meiya Liu[a], Qunfeng Zhang[a,*,1], Jianyun Ruan[a,1]

[a] Tea Research Institute, Chinese Academy of Agricultural Sciences, Key Laboratory for Plant Biology and Resource Application of Tea, The Ministry of Agriculture, Hangzhou, 310008, China

[b] College of Horticulture, Qingdao Agricultural University, Qingdao Key Laboratory of Genetic Improvement and Breeding in Horticultural Plants, Qingdao, China

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ABSTRACT

Widely distributed in tea plants, the flavonoid flavonol and its glycosylated derivatives have important roles in determining tea quality. However, the biosynthesis and accumulation of these compounds has not been fully studied, especially in response to nitrogen (N) supply. In the present study, 'Longjing 43' potted tea seedlings were subjected to N deficiency (0g/pot), normal N (4g/pot) or excess N (16g/pot). Quantitative analyses using Ultra Performance Liquid Chromatography-Triple Quadrupole Mass Spectrometry (UPLC-QqQ-MS/MS) revealed that most flavonol glycosides (e.g., Quercetin-3-glucoside, Kaempferol-3-rgalactoside and Kaempferol-3-glucosyl-rhamnsoyl-glucoside) accumulated to the highest levels when treated with normal N. Results from metabolomics using Gas Chromatography-Mass Spectrometer (GC-MS) suggested that the levels of carbohydrate substrates of flavonol glycosides (e.g., sucrose, sucrose-6-phosphate, D-fructose 1,6-bisphosphate and glucose-1-phosphate) were positively correlated with flavonol glycoside content in response to N availability. Furthermore, Quantitative Real-time PCR analysis of 28 genes confirmed that genes related to flavonoid (e.g., *flavonol synthase 1*, *flavonol 3-O-galactosyltransferase*) and carbohydrate (e.g., *sucrose phosphate synthase, sucrose synthase and glucokinase*) metabolism have important roles in regulating the biosynthesis and accumulation of flavonol glycosides. Collectively, our results suggest that normal N levels promote the biosynthesis of flavonol glycosides through gene regulation and the accumulation of substrate carbohydrates, while abnormal N availability has inhibitory effects, especially excess N.

1. Introduction

Flavonols and related flavonoids are among the most important polyphenolic compounds found in tea (*Camellia* sinensis L.O. Kuntze) (Jiang et al., 2015). Many flavonol derivatives (e.g., myricetin, quercetin and kaempferol) are glycosylated to form stable flavonol glycosides (FGs) through interactions with sugar molecules (e.g., glucose, galactose and rutinose) (Price et al., 1998). At present, more than 2000

Abbreviations: FGs, flavonol glycosides; N, nitrogen; C, carbon; UPLC-QqQ-MS/MS, Quantitative analyses using Ultra Performance Liquid Chromatography-Triple Quadrupole Mass Spectrometry; GC-MS, Gas Chromatography-Mass Spectrometer; qRT-PCR, quantitative real-time PCR; M-GRh, myricetin-3-rhamnosyl-glucoside; M-Ga, myricetin-3-galactoside; M-G, myricetin-3-glucoside; Q-GaRhG, quericetin-3-glucosyl-rhamnosyl-galactoside; Q-GRhG, quercetin-3-glucosyl-rhamnosyl-glucoside; Q-GaRh, quercetin-3-rhamnosylgalactoside; Q-GRh, quercetin-3-rhamnosylglucoside; Q-Ga, quercetin-3-galacoside; Q-G, quercetin-3-glucoside; K-GaRhG, kaempferol-3-glucosyl-rhamnosyl-galactoside; K-GRhG, kaempferol-3-glucosyl-rhamnsoyl-glucoside; K-GaRh, kaempferol-3-rhamnosylgalactoside; K-GRh, kaempferol-3-rhamnosylglucoside; K-Ga, kaempferol-3-galactoside; K-G, kaempferol-3-glucoside; PAL, phenylalanine ammonia-lyase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3β-hydroxylase; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′5′-hydroxylase; FLS1, flavonol synthase 1; DFR, dihydroflavonol reductase; ANS, anthocyanin synthase; ANR1, anthocyanidin reductase 1; F3GlcT, flavonol 3-O-glucosyltransferase; F3GalT, flavonol 3-O-galactosyltransferase; UF3GT, flavonoid 3-O-glycosyltransferase; UF7GT, flavonoid 7-O-glycosyltransferase; GS, glutamine synthetase; GOGAT, glutamate synthase; GDH, glutamate dehydrogenase; PCK, phosphoenolpyruvate carboxykinase; PK, pyruvate kinase; PGmu, phosphoglycerate mutase; SUS, sucrose synthase; SPS, sucrose phosphate synthase; PFK, phosphofructokinase; TPS, trehalose-phosphatesynthase; GK, glucokinase; MDH, malate dehydrogenase; ACCase, acetyl-coenzyme A carboxylase; Asp, aspartic acid; Ser, serine; Glu, glutamic acid; Gly, glycine; His, histidine; Arg, arginine; Thr, threonine; Ala, alanine; Pro, proline; Cys, cysteine; Tyr, tyrosine; Thea, L-theanine

* Corresponding author. Tea Research Institute, Chinese Academy of Agricultural Sciences, South Meiling Road 9, Hangzhou, Zhejiang, 310008, China.
E-mail address: hill@tricaas.com (Q. Zhang).

[1] Equal senior authorship.

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different glycosides have been identified, and about 19 flavonol 3-O glycosides have been reported in green tea and fermented tea. Fourteen of these flavonol 3-O glycosides play important roles in determining the flavor of tea soup (Scharbert et al., 2004). They not only produce dry and smooth astringency sensations in the mouth, but they also increase the bitterness of tea soup by strengthening the bitter taste of caffeine (Scharbert et al., 2004). Studies have also shown that FGs are the main components of green tea soup and that they give color in the form of water-soluble yellow pigments (Dai et al., 2011). Furthermore, FGs play important roles in the color modification, antioxidative, uvioresistant, antihypertensive and antibacterial properties of plants (Pitura and Arntfield, 2018).

Fertilization is an effective way to supply nutrients for tea plants, and it is also an important factor to improve the yield and quality of tea. Nitrogen (N), either from organic or mineral fertilization, is critical for the growth and development of tea plants (Bethke et al., 2007). Olsen et al. (2009) demonstrated that the accumulation of FGs (e.g., flavonol-3-glucoacyl-7-rhamnoside, flavonol-3,7-dirhamnoside and flavonol-3-rutinoside-7-rhamnoside) in *Arabidopsis thaliana* could be induced by N deficiency under low temperature. Similarly, Kováčik and Klejdus (2014) showed that N deficiency significantly increased flavonoid content in *chamomile* leaves and roots, but inhibited the accumulation of flavonols and FGs. In grapes, low N significantly increased the content of anthocyanins, having the greatest impact on the derivatives delphinidin and ptunidin, while sustained N deficiency led to an increase in FG content (Soubeyrand et al., 2014). Additionally, many studies have found that N deficiency triggers the expression of genes involved in flavonoid synthesis, thus promoting the accumulation of flavonoids in most of plants (Olsen et al., 2008; Løvdal et al., 2010).

In tea plants, many of the carbon-containing compounds produced by photosynthesis are used to synthesize protein under N sufficient or excess conditions, which restricts the conversion of some sugars to polyphenols and results in an overall decrease of these compounds. Amino acids and polyphenols, produced by primary and secondary metabolism, respectively, collectively represent an important N and carbon (C) pool in tea plants (Ruan et al., 2010). Increases in N availability can significantly increase the accumulation of amino acids, impact carbon fixation and reduce the accumulation of catechin. The research of Strissel et al. (2005) has shown that under high N conditions, the activities of enzymes related to flavonoid synthesis (e.g., phenylalanine ammonia-lyase, PAL) were down-regulated and flavonoid synthesis was reduced in apple leaves. Ruan et al. (2010) studied the effects of different N levels on C and N metabolism in hydroponic tea seedlings. Their results showed that high N application significantly reduced the total phenolic content of tea shoots, while at the same time, increasing free amino acid content. Yang et al. (2013) found that excess N application decreased the content of catechin.

Although there is significant influence of N on tea plants, the effects of N supply on FG composition, content and biosynthesis have not been systematically studied. To address this, the present study aimed to elucidate the regulatory mechanisms underlying flavonoid biosynthesis and accumulation in tea plants by analyzing changes in FG content and gene expression in response to N availability. Our results provide a theoretical framework for improving nitrogen use efficiency and enhancing tea quality.

2. Material and methods

2.1. Plant materials and growth conditions

Each of four five-year-old 'Longjing 43' tea plant (*Camellia sinensis* (L.) O. Kuntze, a premium clone for green tea) were planted in a pot. The pots (45 cm in inside diameter, 50 cm in depth) filled with commercial growth medium consisting of perlite, vermiculite, and peat (6:3:1, v:v:v) were placed in a greenhouse under natural light, ambient temperature and relative humidity of 70% at the Tea Research Institute, Chinese Academy of Agricultural Sciences in Hangzhou, Zhejiang province (N 30°10′, E 120°5). Potted tea plant treated with three N levels (N1: 0g/pot; N2: 4g/pot; N3: 16g/pot) and each 5 pots as a treatment. The N fertilizer was urea [$CO(NH_2)_2$, nitrogen content 46.4%], and it was split into three seasonal applications (30% in May 2016, after spring tea harvest; 40% in August 2016, before autumn tea and 30% in February 2017, before spring tea). Phosphorus and potassium were supplied, respectively, using superphosphate [$Ca(H_2PO_4)_2$, containing 13% P_2O_5] and potassium sulfate [K_2SO_4, containing 51% K_2O], with the concentrations of the latter two held constant. Three independent biological replicates were used for each condition. Shoots, two expanding leaves and one bud were collected on May 24, 2017. Plant samples were frozen immediately in liquid nitrogen and stored at −80 °C for analysis.

2.2. Quantitative determination of flavonol glycosides

FGs were extracted according to the method described by Zhang et al. (2014). FG standards (myricetin-3-galactoside/glucoside, quercetin-3-glucoside/galactoside, quercetin-3-O-rhamnosyl-D-glucose, kaempferol-3-O-glucoside/galactoside and kaempferol-3-rhamnosyl-D-glucose) were purchased from Sigma (Sigma-Aldrich Co., Louis, MO). Unless otherwise noted, ultrapure Milli-Q water was used in all experiments.

The FG fraction was quantitatively determined by reference (Vrhovsek et al., 2012) and was detected by UPLC-QqQ-MS/MS (ultra-performance liquid chromatography-triple quadrupole tandem mass spectrometry). Separation was performed using a Waters Acquity HSS T3 column (1.8 μm, 100 mm × 2.1 mm, Milford, MA, USA). The ratio of mobile phase A (0.1% aqueous formic acid) and mobile phase B (acetonitrile mixed with 0.1% formic acid) were carried out according to a previously described elution procedure (Vrhovsek et al., 2012). For all measurements, the column temperature and the sample temperature were set at 40 °C, 6 °C, respectively. The flow rate was 0.4 mL/min, 2 μL was injected and the UV detection wavelength were 370 nm.

Mass spectrometry was performed using a Waters Xevo TQ mass spectrometer (Milford, MA, USA) using an ESI source positive ion mode with a source temperature of 150 °C, a desolvation temperature of 500 °C, a cone gas flow rate of 50 L/h, and a solvent removal gas. The flow rate was 800 L/h. Commercially purchased FG standards were quantified using an optimized MRM model (Wang et al., 2015).

2.3. Upstream metabolite analysis based on GC-MS

Sample preparation and derivatization protocols were mainly based on methods established in the literature (Ge et al., 2018; Zhang et al., 2018a). Water, methanol, pyridine, n-hexane, methoxylamine hydrochloride (97%), BSTFA (Bis(trimethylsilyl)trifluoroacetamide) with 1% TMCS (Chlorotrimethylsilane), trichloromethane and L-2-chlorophenylalanine were HPLC grade and purchased from CNW Technologies GmbH (Düsseldorf, Germany), Sinopharm (Shanghai, China), Hengchuang Bio-technology (Shanghai, China), respectively. The samples were analyzed on Agilent 7890B-5977A GC-MSD with ADB-5MS fused-silica capillary column (30 m × 0.25 mm × 0.25 μm, CA, USA). Carrier gas was helium (> 99.999%) at a constant flow rate of 1 mL/min 1 μL sample was injected at 260 °C in splitless mode, and analyzed according to the method described by Chen et al. (2017), The initial oven temperature (60 °C) was ramped to 125 °C at a rate of 8 °C/min, then to 210 °C at a rate of 4 °C/min, increased to 270 °C at a rate of 5 °C/min, finally to 305 °C at a rate of 10 °C/min, and kept 305 °C for 3 min. The temperature of MS quadrupole was set to 150 °C and ion source (electron impact) was at 230 °C.

Total ion chromatograms (TIC) were processed using ChromaTOF software (version 4.34, LECO, St Joseph, MI) and Chemstation (version E.02.02.1431, Agilent, USA). To obtain the metabolic differences among three N levels, a principle component analysis (PCA) and

orthogonal partial least-squares-discriminant analysis (OPLS-DA) were performed. Variable importance in projection (VIP) values from OPLS-DA were carried out as a coefficient of metabolite selection (VIP > 1.0). One-way analysis of variance (ANOVA) from SPSS software (version 20.0, Chicago, IL, USA) was used for statistical and test of the significance of difference was further performed with Student's *t-test* ($P < 0.05$).

2.4. Measurements of total N and free amino acids

Total N content in mature leaves and new shoot leaves was determined by CN analyzer (Elementar, Germany). Quantitative determination of free amino acids was carried out by AccQ.Tag pre-column derivatization and a reversed-phase high performance liquid chromatography system (Waters 2695) with a tandem DAD detector (Waters 2998) as described previously (Jiang et al., 2013). The mobile phase A/B/C was 10% AccQ.Tag buffer, 60% acetonitrile, and 100% Milli-Q water, respectively. The mobile phases were operated at a ratio as described by Han et al. (2018). A Waters AccQ.Tag amino acid analysis column (3.9 mm × 150 mm, 4 μm) was used in this method, the column temperature was set at 37 °C, the flow rate was set at 1 mL/min, and 10 μL was injected. The amino acid standard curve function is shown in Table S1.

2.5. Quantitative real-time PCR analysis

Total RNA was isolated using RNAplant Plus kit (Tiangen, China). The total RNA was reverse transcribed by PrimeScriptTM RT reagent Kit (TaKaRa, Japan). Quantitative real-time PCR (qRT-PCR) was performed on an Applied Biosystems 7500 machine (ABI, USA). GAPDH was used as a reference gene and the primer pairs used for qRT-PCR analysis are shown in Table S2. For each gene, qRT-PCR was performed for three independent biological replications and triplicate reactions were performed. Relative expression levels were calculated using formula $2^{-\Delta\Delta Ct}$ method and normal (N1) was set as control (Zhang et al., 2017).

3. Results

3.1. Phenotype and content of total N in shoots of 'Longjing 43' in response to N availability

As shown in Fig. 1A, there were obvious differences in the leaf color of tea shoots in response to N availability. Leaf colors were yellow-green, light-green and dark green under N deficiency, normal N and excess N, respectively. With an increase in N supply, the total N content of new shoots and mature leaves increased gradually, and was higher in new shoots than in mature leaves (Fig. 1B).

3.2. Differences in FG accumulation in response to N availability

FG content was determined by UPLC-QqQ-MS/MS. For the quantitative detection of 10 FG standards, R^2 values were all greater than 0.985, the linear range was between 0.1 and 3.15 μg/mL, LOD (limit of detection) was 0.0002–0.0005 ng, and LOQ (limit of quantification) was 0.0005–0.0015 ng (Table S3). Using the optimized mass spectrometry conditions, we determined that tea shoot FGs were completely eluted between 4.22 and 6.10 min. The corresponding UV chromatogram is shown in Fig. 2, with the retention times of individual components shown in Table S3. The contents of 15 3-O flavonol glycosides in the tea shoots are shown in Table 1, including three myricetin-3-glycosides, six quercetin-3-glycosides and six kaempferol-3-glycosides.

We observed significant differences in tea shoot FG content in response to the three N treatments. The total amount of FG varied between 2078.7 and 2614.6 mg/kg, with the largest accumulation observed in the normal N (N2) treatment group; FG content was significantly lower ($P < 0.05$) in the N excess (N3) group than in the N-limited (N1) or normal N (N2) groups. Quercetin-3-rhamnosylgalactoside (Q-GaRh) and kaempferol-3-glucosyl-rhamnosyl-galactoside (K-GaRhG) were the most abundant of the detected compounds, accumulating to levels between 263.0 and 374.4 mg/kg and 341.5–401.9 mg/kg, respectively. Meanwhile, quercetin-3-glucoside (Q-G) and kaempferol-3-rhamnosylgalactose (K-GaRh) were the least abundant, accumulating to levels between 23.6 and 35.8 mg/kg and 17.1–23.5 mg/kg, respectively.

We also observed differences in the distribution of mono-glycosides (mG), di-glycosides (dG) and tri-glycosides (tG) of myricetins (M), quercetins (Q) and kaempferols (K) in the shoots of tea plants in response to N availability. The content of myricetin glycoside (TM), quercetin glycoside (TQ) and kaempferol glycoside (TK) of new shoots in the N-deficient (N1) group accounted for 19.34%, 40.39% and 40.27% of the total FG content, respectively; 17.83%, 41.37% and 40.80%, respectively, in the normal N (N2) group; and 19.99%, 40.10% and 39.91%, respectively, in the excess N (N3) group. The content of myricetin mono-glycosides, quercetin di-glycosides and kaempferol tri-glycosides were the highest: the ratios accounted for 91.54%, 61.50% and 64.76% in N1, respectively; 92.09% and 59.94% and 64.81% in N2, respectively; and 91.94%, 64.10% and 68.18% in N3, respectively.

The concentrations of nine additional flavonoid metabolites were also determined by UPLC-QqQ-MS/MS (Fig. 3). In general, the levels of these metabolites were lower in the excess N (N3) group than in the normal N (N2) group, with the exception of gallocatechin gallate (GCG). Additionally, proanthocyanidin B (PB), gallocatechin gallate (GCG) and catechin (C) were reduced in the N-limited (N1) group in comparison to N2.

3.3. Changes in upstream substrates of flavonol in response to N availability

Based on differential metabolite analysis by GC-MS, we observed

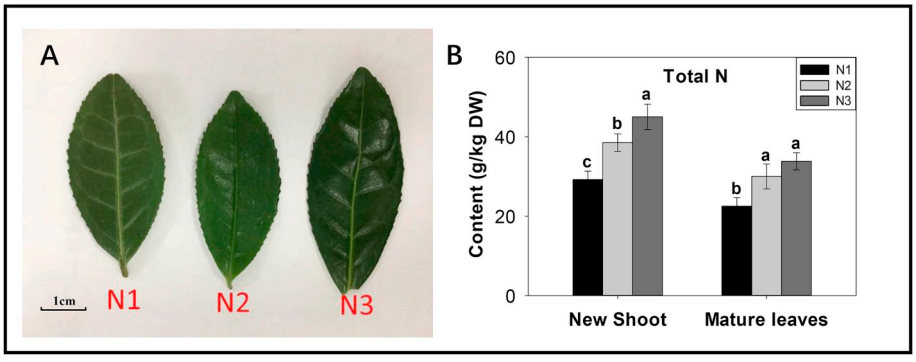

Fig. 1. Phenotype (A) and content of total nitrogen (B) in shoots and mature leaves of 'Longjing 43' in response to N availability.

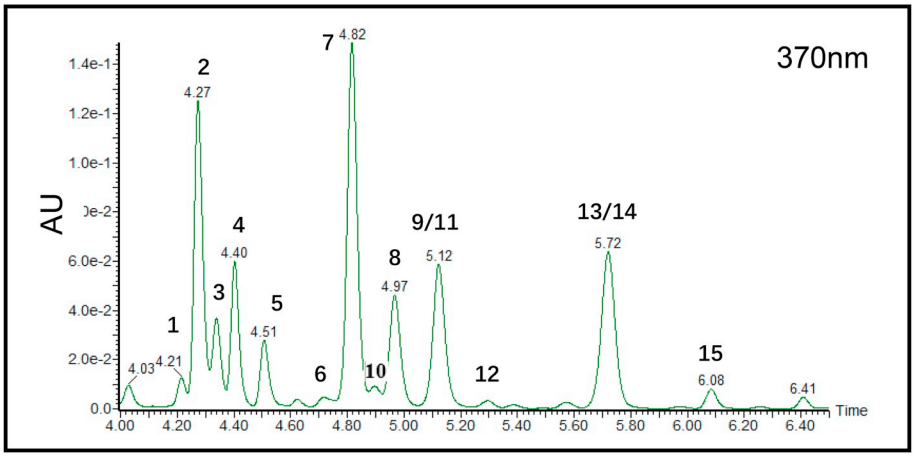

Fig. 2. Chromatogram of flavonol glycosides of 'Longjing 43' tea shoots (wavelength 370 nm). Peak identification: 1, Myricetin-3-rhamnosyl-glucoside; 2, Myricetin-3- galactoside; 3, Myricetin-3-glucoside; 4, Quericetin-3-glucosyl-rhamnosyl-galactoside; 5, Quercetin-3-glucosyl-rhamnosyl-glucoside; 6, Quercetin-3-rhamnosylgalactoside; 7, Quercetin-3-rhamnosylglucoside; 8, Quercetin-3-galacoside; 9, Quercetin-3-glucoside; 10, Kaempferol-3-glucosyl-rhamnosyl-galactoside; 11, Kaempferol-3-glucosyl-rhamnsoyl- glucoside; 12, Kaempferol-3-rhamnosylgalactoside; 13, Kaempferol-3-rhamnosylglucoside; 14, Kaempferol-3-galactoside; 15, Kaempferol-3-glucoside.

Table 1
Content (mg/100 g fresh weight) of flavonol glycosides in shoots of 'Longjing43' under three nitrogen levels (n = 3).

Compounds	OH^{-}[a]	Saccharide[b]	N1	N2	N3
M-GRh	3	di-	45.3 ± 2.5a	40.2 ± 1.3a	37.3 ± 0.12b
M-Ga	3	mono-	215.8 ± 2.0a	210.1 ± 0.5a	187.9 ± 3.0b
M-G	3	mono-	244.7 ± 2.0a	236.0 ± 1.1b	190.3 ± 3.5c
Q-GaRhG	2	tri-	177.5 ± 1.8b	190.3 ± 0.4a	164.6 ± 3.4c
Q-GRhG	2	tri-	122.9 ± 2.5b	145.5 ± 3.9a	116.4 ± 3.7b
Q-GaRh	2	di-	340.9 ± 3.1a	346.6 ± 9.2a	263.0 ± 4.5b
Q-GRh	2	di-	181.8 ± 3.0b	202.5 ± 3.8a	126.0 ± 1.5c
Q-Ga	2	mono-	197.3 ± 2.6a	195.3 ± 0.6a	138.1 ± 1.3b
Q-G	2	mono-	35.8 ± 1.8b	47.7 ± 1.8a	23.6 ± 2.8c
K-GaRhG	1	tri-	401.2 ± 1.9a	401.9 ± 1.9a	341.5 ± 4.6b
K-GRhG	1	tri-	282.9 ± 3.5b	302.1 ± 3.7a	240.3 ± 5.9c
K-GaRh	1	di-	23.5 ± 1.1a	20.4 ± 0.5b	17.1 ± 2.0c
K-GRh	1	di-	52.5 ± 1.7b	55.2 ± 0.2a	32.4 ± 1.9c
K-Ga	1	mono-	247.0 ± 3.5b	282.5 ± 6.9a	166.9 ± 7.5c
K-G	1	mono-	45.5 ± 1.2b	50.2 ± 1.0a	33.3 ± 1.3c
Total			2614.6	2726.5	2078.7

Values shown are means ± SD (N = 3). Different letters within rows indicate a significant difference between the means at $P < 0.05$. Abbreviations: M-GRh, myricetin-3-rhamnosyl-glucoside; M-Ga; myricetin-3-galactoside; M-G, myricetin-3-glucoside; Q-GaRhG, quericetin-3-glucosyl-rhamnosyl-galactoside; Q-GRhG, quercetin-3-glucosyl-rhamnosyl-glucoside; Q-GaRh, quercetin-3-rhamnosylgalactoside; Q-GRh, quercetin-3-rhamnosylglucoside; Q-Ga, quercetin-3-galacoside; Q-G, quercetin-3-glucoside; K-GaRhG, kaempferol-3-glucosyl-rhamnosyl-galactoside; K-GRhG, kaempferol-3-glucosyl-rhamnsoyl-glucoside; K-GaRh, kaempferol-3-rhamnosylgalactoside; K-GRh, kaempferol-3-rhamnosylglucoside; K-Ga, kaempferol-3-galactoside; K-G, kaempferol-3-glucoside.

[a] B-ring hydroxyl number of flavonol glycoside structure.

[b] Number of glycosyl groups.

that most carbohydrates and conjugates showed decreased accumulation in the shoots of tea plants from the N-limited (N1) and excess N (N3) groups compared to the normal N (N2) group. These included 6-deoxy-D-glucose, D-talose, fucose, lyxose, raffinose, ribose, sucrose and tagatose (Table 2). By contrast, D-fructose 1,6-bisphosphate were significantly increased in the N1 and N3 groups relative to N2. Additionally, we observed higher levels of D-altrose in N3 than in N2, but lower levels in N1 than in N2. The opposite was observed for ribulose-5-phosphate. Within the TCA cycle, alpha-ketoglutaric acid, citric acid, isocitric acid, L-malic acid and succinic acid were all significantly decreased in N1 relative to N2. Except for alpha-ketoglutaric acid, these organic acids and derivatives also accumulated to lower levels in N3 than in N2 (Table 2).

Amino acids were measured by GC-MS as biomarkers for the three N treatment groups and were used to correlate C metabolism with N metabolism. The accumulation of amino acids, particularly for glycine, increased with increasing N availability (Table 3). Quantification of amino acids verified the metabolomics results and suggested that glycine is sensitive to N stress. However, theanine, a unique amino acid in tea plants, showed reduced content in N1 (relative to N2); a significant difference was not observed between N2 and N3 (Table 3). Interestingly, γ-aminobutyric acid (GABA) was significantly increased in N1 and N3 (Table 2, $P < 0.01$), suggesting an important role for this signaling molecule under N stress in tea plants.

3.4. qRT-PCR analysis of key genes involved in flavonol and substrate biosynthesis in response to N availability

Compared with the N deficient (N1) group, phenylpropanoid biosynthesis genes were down-regulated in tea shoots supplied with normal (N2) or excess (N3) N (Fig. 4). These included genes encoding phenylalanine ammonia-lyase (*PAL*), chalcone synthase (*CHS*), chalcone isomerase (*CHI*) and flavonoid 3-hydroxylase (*F3H*). The flavonoid 3′,5′-hydroxylase (*F3′5′H*) gene was up-regulated in N3 relative to N2, however. Among the key genes that catalyze branching steps of the catechin and flavonol synthesis pathways, dihydroflavonol 4-reductase (*DFR*), anthocyanidin synthase (*ANS*) and anthocyanidin reductase 1 (*ANR1*) showed a similar trend as *F3′5′H* (i.e., N3 > N1 > N2). However, flavonol synthase 1 (*FLS1*), glycosyltransferases (*F3GalT*, *UF7GT*) and the transcription factor *MYB7-1* were relatively suppressed in N1 and N3 compared with N2 (Fig. 4).

Genes encoding enzymes involved in carbohydrate metabolism were also assessed. The qRT-PCR results showed that phosphoenolpyruvate carboxykinase (*PCK*), pyruvate kinase (*PK*) and phosphoglycerate

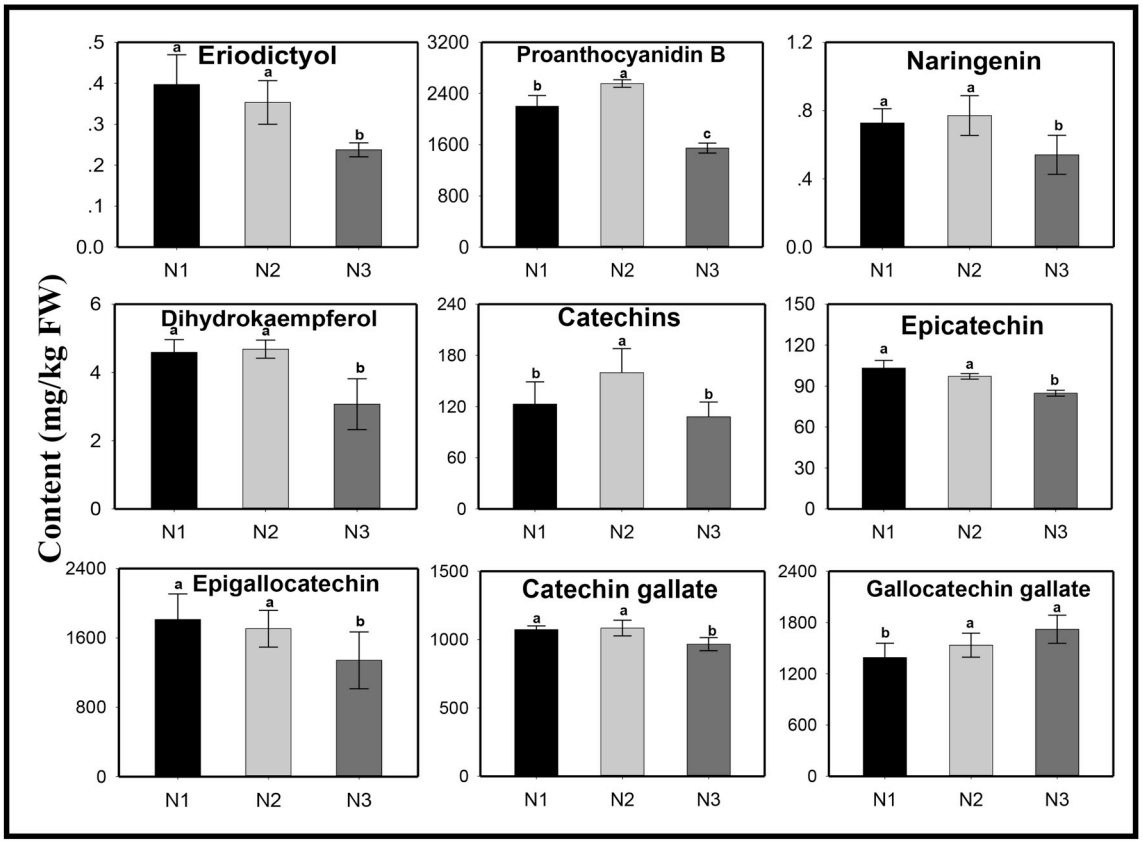

Fig. 3. Quantification of flavonoid compounds in 'Longjing 43' tea shoots in response to N availability.

mutase (*PGmu*) were up-regulated in N1 and N3 relative to N2. In contrast, sucrose synthase (*SUS*), sucrose phosphate synthase (*SPS*), phosphofructokinase (*PFK*), trehalose-phosphate synthase (*TPS*), glucokinase (*GK*) and acetyl-coenzyme A carboxylase (*ACCase*) were all down-regulated in N1 and N3 relative to N2, with larger differences observed between N1 and N2 than N3 and N2 for *SUS*, *SPS*, *PFK* and *TPS*. In addition, malate dehydrogenase (*MDH*) involved in the TCA cycle was found to be up-regulated in N1 and down-regulated in N3 (Fig. 4). Finally, glutamine synthetase (*GS*) and glutamate dehydrogenase (*GDH*), both important for converting inorganic N into amino acids, were up-regulated in N1 and N3 compared to N2, while the gene encoding glutamate synthase (*GOGAT*) was reduced in N1 and N3 compared to N2 (Fig. 4).

4. Discussion

4.1. Influence of N supply on FG accumulation in shoots of 'Longjing 43'

For the perennial leaf-harvested tea plant, the levels and forms of bio-available N strongly impact the biosynthesis of primary and secondary metabolites, leading to differences in the accumulation of compounds that determine the quality and flavor of tea (Huang et al., 2018). Ruan et al. (2010) used different N levels to study the effects on C and N metabolism in hydroponic tea seedlings, which showed that nitrogen use efficiency was significantly negatively correlated with the total phenolic content in tea shoots, such that the total phenol content significantly decreased with an increase in available N. Furthermore, their results were validated by field experiments (Yan et al., 2014). Yang et al. (2013) further explored the relationship between catechin content and N application, and higher N levels significantly inhibited the accumulation of catechins. In the present study, most FGs (e.g., Q-G, K-Ga and Q-GRhG) showed higher levels of accumulation in tea plants grown in the presence of normal N levels than in N deficiency and excess N conditions (N2 > N1 > N3, $P < 0.05$, Table 1). Our results are consistent with those of Kováčik and Klejdus (2014), who demonstrated decreased FG content in *Chamomilla* under N deficiency. Based on measurements of flavonoid content (Table 1 and Fig. 3), we observed a reduced flow to secondary C metabolism under N deficiency, including a decrease in FG content. We noted that under such conditions, tea leaves appeared to accumulate less chlorophyll (Fig. 1), which may indicate reduced capacity for photosynthetic CO_2 assimilation and subsequent carbohydrate production. Previous work has shown that accumulation of FGs is strongly dependent on the biosynthesis of sugar substrates of flavonols (Liu et al., 2014). Similarly, others have reported that carbohydrates and intermediate metabolites provide substrates for anthocyanin biosynthesis (Kim et al., 2006; Li et al., 2017). In the present study, carbohydrates and intermediate metabolites (e.g., sucrose, sucrose-6-phosphate, D-fructose 1,6-bisphosphate and glucose-1-phosphate) showed reduced accumulation under N deficiency (relative to normal N). We suggest that this is caused by reduced photosynthetic capacity (i.e., carbon assimilation) under N deficiency (Mohotti and Lawlor, 2002; Dong et al., 2018). Interestingly, trehalose-6-phosphate significantly accumulated in the N-deprived (N1) group. It was previously reported that high Tre6P levels decreased sucrose levels by stimulating nitrate assimilation (Figueroa et al., 2016), indicating Tre6P might play an important role in coordinating C and N metabolism under N deficiency. Zhang et al. (2018a,b) found a significant negative correlation between N metabolism (amino acids) and C metabolism (e.g., carbohydrates, organic acids and flavonoids) in albino tea leaves, suggesting that increased N metabolism has a weakening effect on C metabolism. Similar results are demonstrated in the present study, as evidenced by increases in amino acid content (e.g., Glu, Gly, Ser and Pro, Table 3) and decreases in many carbohydrates and conjugates with increasing N availability (Table 2). In other words, excess N stimulates N metabolism and suppresses C metabolism. Interestingly, the content of GABA was significantly increased under conditions of N deficiency

Table 2
Significantly changed (VIP > 1 and P-value < 0.05) intracellular metabolites in tea shoots under three nitrogen levels.

Compounds	N1/N2			N3/N2		
	VIP[a]	FC[c]	P-Value[b]	VIP	FC[d]	P-Value
Amino Acid and Derivatives						
Alanine	N	N	N	1.48	1.64	**
Aspartic acid	1.14	0.78	**	N	N	N
Beta-Alanine	1.25	0.77	**	N	N	N
Glutamine	N	N	N	1.38	2.55	*
Glycine	1.40	0.60	**	1.16	1.34	**
Methionine	N	N	N	1.50	4.60	**
Oxoproline	1.27	0.80	*	1.37	2.04	*
Proline	1.27	0.86	*	N	N	N
Threonine	N	N	N	1.43	5.48	**
γ-aminobutyric acid (GABA)	1.44	1.27	**	1.57	3.42	**
Organic Acids and Derivatives						
Alpha-ketoglutaric acid	1.56	0.75	**	N	N	N
Benzoic acid	1.41	0.75	**	1.01	0.88	*
Chlorogenic Acid	1.24	1.26	**	1.35	0.70	**
Citric acid	1.50	0.82	**	1.52	0.62	**
Gallic acid	1.57	1.76	**	1.01	1.09	*
Isocitric acid	1.59	0.57	**	1.34	0.68	**
L-Malic acid	1.57	0.79	**	1.53	0.77	**
Pyruvic acid	1.55	0.62	**	N	N	N
Quinic acid	N	N	N	1.51	0.75	**
Salicylic acid	1.60	0.61	**	1.56	0.47	**
Shikimic acid	1.25	0.92	*	1.54	0.73	**
Succinic acid	1.17	0.91	**	1.52	0.75	**
Carbohydrates and conjugates						
3,6-Anhydro-D-galactose	1.30	0.27	**	N	N	N
6-Deoxy-D-glucose	1.45	0.76	**	1.44	0.75	**
Cellobiose	N	N	N	1.53	0.71	**
D-Altrose	1.52	0.62	**	1.24	1.24	**
D-Fructose 1,6-bisphosphate	1.58	1.26	**	1.51	1.32	**
D-Talose	1.57	0.56	**	1.49	0.62	**
Fucose	1.14	0.78	*	1.27	0.68	**
Glucose-1-phosphate	1.58	0.69	**	1.29	0.82	**
Lyxose	1.52	0.74	**	1.47	0.61	**
Raffinose	1.13	0.67	**	1.16	0.71	**
Ribose	1.59	0.58	**	1.35	0.68	**
Ribulose-5-phosphate	1.12	1.10	*	1.16	0.86	**
Sorbose	1.28	0.68	**	N	N	N
Sucrose	1.23	0.93	**	1.16	0.94	**
Sucrose-6-Phosphate	1.30	0.73	**	N	N	N
Tagatose	1.57	0.48	**	1.51	0.53	**
Trehalose-6-phosphate	1.28	1.21	**	N	N	N

"*" indicates Significant difference (P < 0.05).
"**" indicates extremely significant difference (P < 0.01).
[a] VIP is the variable importance in the projection values from partial least squares discriminant analysis (PLS-DA).
[b] P-value is significant difference in metabolites. "N" indicates no significant difference.
[c] N1/N2 is the ratio of the mean peak intensity in nitrogen deficient relative to nitrogen normal.
[d] N3/N2 is the ratio of the mean peak intensity in nitrogen sufficient relative to nitrogen normal.

and excess, indicating that this signaling molecule may be critical for responding to N stress (Table 2, Fritz et al., 2006). Overall, N deficiency or excess resulted in decreased accumulation of FGs in tea shoots.

Glycosylation is the most frequently observed modification among FGs. In tea shoots, FGs mainly include quercetin glycoside (QG), myricetin glycoside (MG) and kaempferol glycoside (KG). Among these, KG and QG are the largest groups, including 458 chemicals and 363 chemicals, respectively. While some positions are most favored on a flavonoid skeleton in nature. The 3-/7-OH in flavonols and flavanols, 7-OH in flavones, flavanones and isoflavones, as well as 3-/5-OH in anthocyanidins are common glycosylation sites (Hiromoto et al., 2015). At present, all FGs found in tea are 3-O glycosides, but based on the length of the sugar chain, flavonoid O-glycosides are divided into monosaccharides, disaccharides, trisaccharides and tetrasaccharides

Table 3
Content (mg/100 g fresh weight) of free amino acids in shoots of 'Longjing43' under three nitrogen levels (n = 3).

Amino acids	N1	N2	N3
Asp	3.20 ± 0.05b	3.90 ± 0.10a	4.38 ± 0.09a
Ser	2.42 ± 0.98c	4.36 ± 0.27b	6.05 ± 0.68a
Glu	5.53 ± 0.01c	8.38 ± 0.96b	12.32 ± 0.13a
Gly	3.55 ± 0.18c	4.82 ± 0.26b	9.41 ± 0.83a
His	5.34 ± 0.05 ab	6.49 ± 2.22a	6.05 ± 0.22b
Arg	246.22 ± 10.78a	255.48 ± 3.16a	257.70 ± 0.47a
Thr	52.85 ± 0.03b	55.06 ± 0.32b	59.29 ± 2.83a
Ala	6.01 ± 0.06b	6.08 ± 0.49b	6.76 ± 0.76a
Pro	7.40 ± 0.08c	10.25 ± 0.38b	13.24 ± 0.48a
Cys	8.18 ± 0.35b	10.06 ± 0.01a	10.22 ± 0.38a
Tyr	56.44 ± 4.18b	82.22 ± 6.40a	86.68 ± 3.87a
Thea	472.24 ± 4.72b	565.28 ± 18.38a	573.72 ± 7.40a

Values shown are means ± SD (N = 3). Different letters within rows indicate a significant difference between the means at P < 0.05. Asp, aspartic acid; Ser, serine; Glu, glutamic acid; Gly, glycine; His, histidine; Arg, arginine; Thr, threonine; Ala, alanine; Pro, proline; Cys, cysteine; Tyr, tyrosine; Thea, L-theanine.

(Wang et al., 2015; Lin et al., 2008). In this work, we showed that myricetin-3-monoglycoside (mM), quercetin-3-diglycoside (dQ) and kaempferol-3-triglycoside (tK) accounted for the largest proportion of their respective flavonol components (Table 1), a trend that did not change in response to N supply, indicating that nutritional factors may have little effect on the glycosylation state of FGs. The B rings of MG, QG and KG are linked with trihydroxy, dihydroxy and monohydroxy groups, respectively. Studies have confirmed that the B-ring hydroxyl number on an FG is positively correlated with its antioxidant activity: MG > QG > KG (Zhang et al., 2017). It is speculated that the degree of glycosidation may be affected by the hydroxylation of the B-ring structure, and that flavonols with high antioxidant activity are more likely to synthesize glycosides with a lower degree of glycosylation. In addition, glycosylation enhances molecular polarity and water solubility, making flavonols more stable in plant vacuoles (Lin et al., 2010; Cao et al., 2018). This indicates that the distribution form of FGs may be related to the storage stability of glycosides in plant vacuoles.

4.2. Effect of N supply on FG biosynthesis in shoots of 'Longjing 43'

The synthesis of flavonol mainly depends on the flavonoid metabolic pathway. Previous studies have found that N deficiency induced the expression of flavonoid biosynthetic genes and related transcription factors in plants (Fan et al., 2016). Similar results have been observed in this study, the expression of genes involved in the phenylpropanoid pathway (*PAL, CHS* and *CHI*) were up-regulated (Fig. 4). PAL, the first enzyme in the flavonoid pathway, which regulates primary metabolic fluxes of C into this pathway (Xu and Yang, 2009), was especially up-regulated, indicating that lower N promoted the biosynthesis of flavonoids in tea plants (Kováčik et al., 2009; Zhou et al., 2016). The expression of *FLS1* suggests that flux is directed through the flavonol synthesis branch, thus controlling the composition and content of flavonols (Cao et al., 2018). Multiple studies have demonstrated that expression of *FLS* is regulated by single or complex transcription factors such as *MYB*, *bHLH*, *AtMYB111* and *AtMYB12* in *Arabidopsis thaliana* (Mehrtens et al., 2005; Stracke et al., 2007). These transcription factors, as well as *VvMYB1* in *Vitis vinifera* (Deluc, 2006), may activate the *AtFLS* and *VvFLS* promoter, respectively, promoting the biosynthesis of flavonols. The study of Zhao et al. (2013) indicated the genes of MYB7 subfamily may be involved in flavonoid metabolism. Chen et al. (2015) found that the transcription factor *MYB7-1* has high homology with *AtMYB111/12* and *VvMYB1*, and it was confirmed in tobacco that *MYB7-1* positively regulates *NtFLS* production, further content of flavonol was accumulated. In the present study, the expression level of

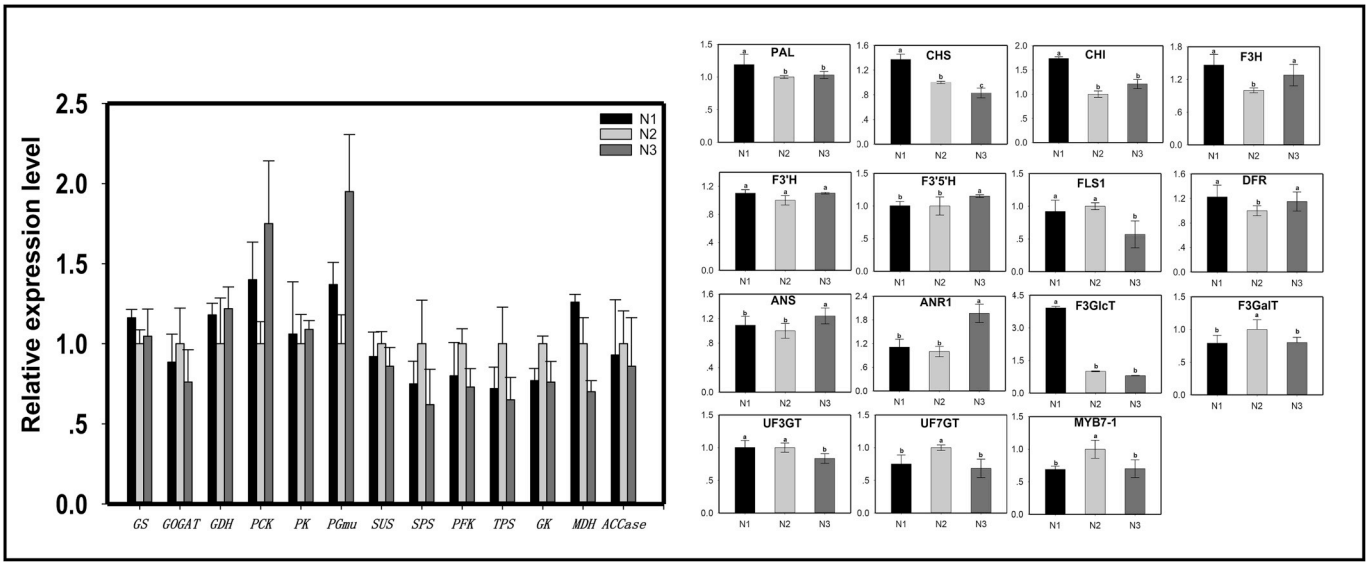

Fig. 4. Quantitative RT-PCR results of genes involved in flavonoid, nitrogen and carbon metabolism in the shoots of 'Longjing' tea in response to N availability. Standard error of the mean for three technical replicates is shown. qRT-PCR amplification of the GAPDH gene was chosen as an internal standard. PAL, phenylalanine ammonia-lyase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3β-hydroxylase; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′5′-hydroxylase; FLS1, flavonol synthase 1; DFR, dihydroflavonol reductase; ANS, anthocyanin synthase; ANR1, anthocyanidin reductase 1; F3GlcT, flavonol 3-O-glucosyltransferase; F3GalT, flavonol 3-O-galactosyltransferase; UF3GT, flavonoid 3-O-glycosyltransferase; UF7GT, flavonoid 7-O-glycosyltransferase; GS, glutamine synthetase; GOGAT, glutamate synthase GDH, glutamate dehydrogenase PCK, phosphoenolpyruvate carboxykinase; PK, pyruvate kinase; PGmu, phosphoglycerate mutase; SUS, Sucrose synthase; SPS, sucrose phosphate synthase; PFK, phosphofructokinase; TPS, trehalose-phosphate-synthase; GK, glucokinase; MDH, malate dehydrogenase; ACCase, acetyl-coenzyme A carboxylase.

MYB7-1 was analyzed in shoots of 'Longjing43'. Similar to the results of Chen et al. (2015), our data suggested that the expression of *CsMYB7-1* is increased under normal N availability, leading to the induction of *CsFLS1* and the accumulation of FGs. Therefore, *CsFLS1* might be positively regulated by *CsMYB7-1* in response to N supply. Modification by glycosylation is usually the last step in the production of many secondary metabolites such as flavonols and anthocyanins, and plays an important role in determining the diverse functions of polyphenolic compounds in plants as well as influencing their solubility and stability (Yoshida et al., 2000; Jones and Vogt, 2001). Huang et al. (2018) found that the gene encoding UDP-flavonoid glycosyltransferase (*UFGT*) is down-regulated in response to increased N supply. Our results also showed that the orthologous *UFGT* gene (*UF3GT, UF7GT*) is down-regulated under excess N, which raises the possibility that UFGT activity may be inhibited due to reduced sugar substrate content under conditions of high N availability. Cui et al. (2016) confirmed that *CsF3GlcT* and *CsF3GalT* are specifically involved in the biosynthesis of flavonol 3-O glucosides and flavonol 3-O galactosides, respectively, and that there is a strong positive correlation between *CsUGTs* and the accumulation of flavonol 3-O-monoglycoside. However, from our results demonstrating that the expression of *CsF3GalT* was positively correlated with the accumulation of flavonol 3-O galactosides under excess N, it is speculated that compared with *CsF3GlcT*, *CsF3GalT* is more likely to be regulated by *CsMYB7-1*, further influencing the accumulation of FGs.

A schematic flow chart of the biological pathways involved in regulating FG biosynthesis in response to N supply and the corresponding changes in the expression of key genes are shown in Fig. 5 and Fig. 4, respectively. Most genes are related to carbohydrate metabolism, including the glycolytic pathway, metabolism of sucrose and pyruvate, and the TCA cycle. In the pathway of sucrose, the expression of genes was suppressed under abnormal N availability, including *SUS*, *SPS* and *GK*, which might directly lead to result in a reversible conversion rate being reduced, including sucrose to fructose and UDP to UDP-glucose (Baroja-Fernández et al., 2012). ATP, intermediates and reducing equivalents were provided through Glycolysis pathway for the synthesis of most compounds (e.g., amino acids, phenylpropanoids, fatty acids and isoprenoids) (Voll et al., 2009). In our study, the expression levels of *PGmu* and *PK* were significantly up-regulated under N deficiency or excess, suggesting that the biosynthetic pathway from hexose to pyruvate is activated. The observed changes in accumulation of TCA cycle organic acids (i.e., L-malic acid, citric acid, isocitric acid, pyruvic acid and succinic acid) under N deficiency or excess conditions (Table 2) may be attributable to a feedback mechanism, such that the TCA cycle was promoted and more C skeletons were used for the synthesis of amino acids. Moreover, the expression levels of genes involved in N metabolism (i.e., *GS* and *GDH*, Fig. 4) were up-regulated in response to N deficiency or excess, indicating that the GS/GOGAT cycle was also enhanced and more C from the TCA (namely ketoglutaric acid) was used for re-assimilation of ammonium (Satou et al., 2014). Therefore, we propose two mechanisms to explain the reduction in FG accumulation under N deprivation or excess N availability. On the one hand, the synthesis of carbohydrates is inhibited, resulting in limited sugar substrates such as UDP-glucose and hexoses. On the other hand, such conditions may promote flux of C skeletons to the TCA cycle and the GS/GOGAT cycle for the re-assimilation of ammonium.

5. Conclusion

In this study, fifteen flavonol glycosides were quantified in response to N availability. Most FGs (e.g., Q-G, K-Ga and Q-GRhG) showed the highest level of accumulation when tea plants were supplied with normal levels of N. Moreover, substrate metabolites of FG (e.g., sucrose, sucrose-6-phosphate, D-fructose 1,6-bisphosphate and glucose-1-phosphate), FG biosynthesis genes (e.g., *CsF3GalT, CsFLS1*) were identified as playing critical roles in regulating the biosynthesis and accumulation of FGs in response to N supply.

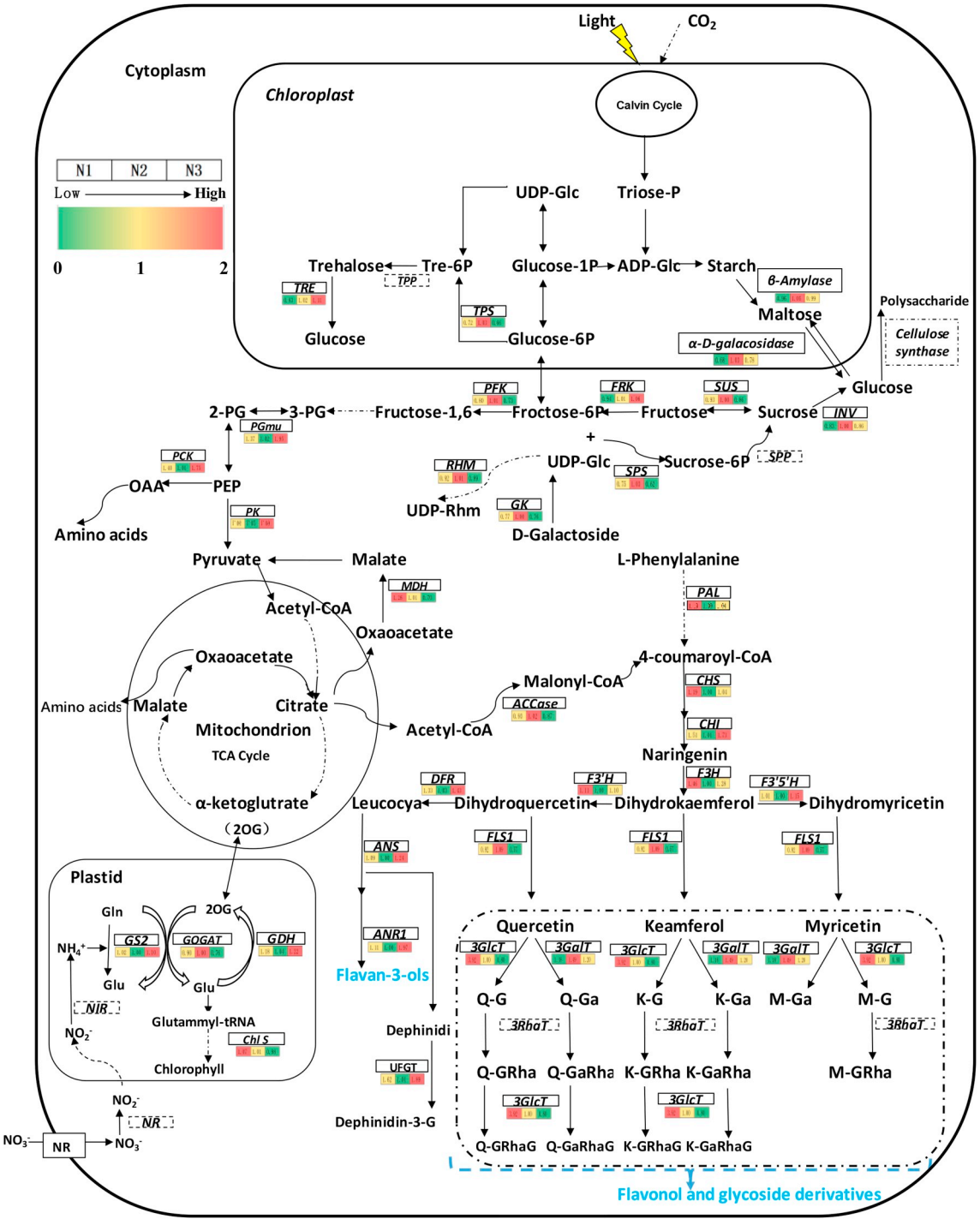

Fig. 5. Schematic representation of biological pathways involved in the regulation of flavonol glycosides biosynthesis in shoots leaves of 'Longjing 43'. The expression of thirty-three genes were integrated, and were indicated either in red (up-regulated) or green (down-regulated) under in response to N availability, as represented by the three boxes (left, N deficiency; middle, normal N; right, excess N). Glu, glutamic acid; Gln, Glutamine; OAA, oxaloacetic acid; PEP, phosphoenolpyruvate; 2-PG, 2-monophosphoglycerate; 3-PG, 2-monophosphoglycerate; Tre-6P, trehalose-6-phosphate; UDP-Glc, Uridine 5′-diphosphoglucose; ADP-Glc, adenosine diphosphate glucose; Dephinidin-3-G, delphinidin3-glucoside; TRE, trehalase; TPP, trehalose-6-phosphate phosphatase; FRK, fructokinase; INV, sucrose invertase; SPP, sucrose-6-phosphate phosphatase; RHM, rhamnose synthase; 3RhaT, UDP－Rhamnose synthase; Chl S, Mg-chelatase D subunit. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Author contributions statement

FD, QZ and JR designed the research; FD performed most the experiments and wrote the paper; QZ helped to analyze the metabolome data; JH and ML discussed the data; YS provided resources; QZ and JH revised the manuscript; JR and ZQ provided fund support. All the authors have reviewed and approved the manuscript.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.02.017.

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