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ORIGINAL ARTICLE

Transcriptome analysis using RNA-Seq revealed the effects of nitrogen form on major secondary metabolite biosynthesis in tea (*Camellia sinensis*) plants

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Abstract
The effects of the different forms of N, ammonium (NH_4^+) and nitrate (NO_3^-), on secondary metabolite biosynthesis in tea plants are well known. However, the mechanisms of underlying these effects are not well understood. This study generated transcriptomic profiles of *Camellia sinensis* treated with different N forms for 5 min and 96 h using RNA-Seq. Analysis of differentially expressed genes (DEGs) showed that the regulated DEGs involved in the secondary pathways in the NH_4^+-treatment were more abundant than those in the NO_3^-- and NH_4^++NO_3^--treatments, demonstrating the preference of tea plants for NH_4^+ at the gene level. Expression analysis at different treatment times showed that increasing treatment time changed not only the number of DEGs involved in secondary metabolism but also the pathways of secondary biosynthesis. In addition, only a few DEGs involved in secondary metabolism were regulated in more than one treatment. These results suggested that the secondary biosynthesis pathways regulated by N were dependent on the N form and treatment time. The analysis of DEGs related to transport and N metabolism further confirmed this conclusion. Moreover, the change of DEGs in NO_3^--treated tea plants suggested increase of treatment time might attenuate the flavonoid biosynthesis and enhance the theanine and caffeine biosynthesis. The identification of differentially expressed transcription factors revealed the possible crosstalk between the secondary metabolic pathways in *C. sinensis*. These results help facilitate an understanding of the effects of N on tea quality and represent a valuable reference for the rational application of N fertiliser on tea plantations.

Keywords Transcriptome analysis · Secondary biosynthesis · RNA-Seq · Tea · Nitrogen form

Introduction

Consumed by more than one-half of the population, tea has been a popular non-alcoholic beverage throughout the world, with approximately 3 billion cups being consumed daily worldwide. Many medicinal properties of tea, such as its antisenile, anticaries and anticarcinogenic activities, have been verified and described in humans (Chen and Chen 2012; de Mejia et al. 2009; Li and Silva 2011). The aroma, nutritional value and health-promoting functions of tea are mostly determined by a mass of secondary metabolites, consisting of three major characteristic constituents, including flavonoids, free amino acids (mainly theanine) and caffeine. Flavonoids are derived from phenylalanine, including catechins, flavonols, flavones, isoflavones, flavanols, flavanones

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and anthocyanidins, which play important roles in antioxidant activity and protection against ultraviolet light (Dixon and Pasinetti 2010). Theanine is a unique free amino acid, that accounts for approximately half of the total amino acids in tea, providing tea a unique taste known as "umami" (Li and Silva 2011; Yamaguchi and Ninomiya 2000). Caffeine is one of the major alkaloids occurring abundantly in tea and widely used as a stimulant (Hewavitharanage et al. 1999).

These three main components of tea are affected by nutrient availability and environmental factors. Nitrogen (N) is a key element for tea plants, and it is most concentrated in harvestable young shoots (Ruan et al. 2007a, b), thus the utilisation of N fertilisers is widely practised to increase tea plant productivity in tea plantations (Kamau et al. 2008). The N source affects tea plant growth and regulates the synthesis of characteristic constituents in the leaves (Ruan et al. 2007a, b; Chen and Chen 2012; Fan et al. 2015). Decreased total N concentration has been attributed to decreases in the concentrations of amino acids and caffeine in mature tea plant leaves (Li et al. 2016). Ammonium (NH_4^+) and nitrate (NO_3^-) are two key N sources that can be absorbed by plants in soils. Tea has been reported to prefer NH_4^+ over NO_3^- as its N source, and is well adapted to high NH_4^+ environments by accumulating high amounts of NH_4^+ in roots. The much higher accumulation of NH_4^+ compared with NO_3^- contributes to the considerably higher accumulation of amino acids, caffeine and chlorophyll content and the increase in biomass in NH_4^+-fed plants compared with NO_3^--fed plants (Ruan et al. 2007a, b, 2016; Li and Silva 2011; Fan et al. 2015).

The transcriptome dataset of tea plants have been obtained from deep sequencing, and many genes of major metabolic pathways have been discovered (Shi et al. 2011). The gene expression profiles of different tea plant tissues have been used to analyse the possible crosstalk in gene regulation between the secondary metabolite biosynthetic pathways in *Camellia sinensis* (Li et al. 2015). Using RNA-Seq and amino acid measurements, Li et al. (2017) found 196 and 29 common DEGs in tea plant roots and leaves, respectively, in two varieties of *C. sinensis* in response to the NH_4^+. However, we still know little about the molecular mechanisms of N effects on secondary metabolite biosynthesis in tea plants. Using RNA-Seq technology, this study elucidated the molecular basis of the NH_4^+ preference and characterised the global expression profiles of genes involved in the secondary metabolism of tea plants treated with different N fertilisers, providing comprehensive insight into understanding the relationship of the N form and quality of tea and representing a valuable reference for the rational application of N fertiliser in tea plantations.

Materials and methods

Plant material

Two-year-old tea plants 'Longjing 43' were grown in the Jiangsu Province Academy of Agricultural Sciences, Nanjing, China. The rooted cuttings were washed and transplanted to nutrient solutions containing the macronutrients P (0.03 mmol L^{-1}), K (0.33 mmol L^{-1}), Ca (0.27 mmol L^{-1}) and Mg (0.13 mmol L^{-1}) and the micronutrients B (3.33 µmol L^{-1}), Mn (0.5 µmol L^{-1}), Zn (0.33 µmol L^{-1}), Cu (0.07 µmol L^{-1}), Mo (0.17 µmol L^{-1}) and Fe (2.10 µmol L^{-1}) as an EDTA salt. The plants were cultivated in the above nutrient solutions for 1 week and maintained in a growth chamber under a day/night ratio of 16/8 h. The relative humidity was kept at 80%, and the temperature was maintained at approximately 25 °C.

Tea plants were incubated in CK, NH_4^+, NO_3^- and NH_4^+:NO_3^- = 1:1 (3 mmol L^{-1}) solutions. After termination of uptake, six plants from each treatment were collected at 0 min, 5 min and 96 h. The plants were washed three times in deionized water and separated into roots stems and mature leaves and shoots. They were immediately frozen in liquid nitrogen and stored at − 70 °C.

RNA preparation

Total RNA was prepared using the Plant RNA Extraction Kit (Zoonbio Biotechnology, China) for eight mixed samples, including buds, mature leaves, stems and roots, which had been shock-frozen in liquid nitrogen and stored at − 70 °C. The integrities and concentration of the tea plant RNA samples were determined using the Agilent 2100 Bioanalyzer System and the RNA Nano 6000 Assay Kit (Agilent Technologies, CA, USA).

Library preparation, sequencing and assembly

Total RNA (3 µg) from each sample was used for preparation of the cDNA library for transcriptome sequencing. Following the manufacturer's instructions, the sequencing libraries were generated using the RNA Library Prep Kit for Illumina (NEB, USA). Index codes were added to attribute sequences to each sample. The cDNA library quality was detected using an Agilent 2100 Bioanalyzer.

For cluster generation, the TruSeq PE Cluster Kit v3-cBot-HS (Illumina) was used according to the manufacturer's protocol. Next, the library preparations were sequenced on an Illumina Hiseq 2000 platform, and 150 bp paired-end reads were obtained.

The raw reads in fastq format were first filtered by in-house Perl scripts. High-quality clean reads were obtained by removing adaptor sequences, reads containing poly-Ns and low-quality reads. The sequence duplication level of the clean data and the Q20, Q30 and GC-contents were calculated. The following analyses were based on high-quality clean data.

The files from all libraries were separated into two single large files (left.fq file and right.fq file). Transcriptome assembly was completed based on these two files using Trinity, with the "min kmer cov" set to two and all other parameters set to default (Grabherr et al. 2015).

Gene functional annotation

All Illumina-assembled unigenes related to putative gene descriptions, gene ontology (GO) terms, conserved domains and putative metabolic pathways longer than 200 bp were annotated based on those of similar sequences from the Nr(NCBI non-redundant protein sequences), Nt (NCBI non-redundant nucleotide sequences), Pfam(Protein family), KOG/COG (Clusters of Orthologous Groups of proteins), Swiss-Prot (a manually annotated and reviewed protein sequence database), KO(KEGG Ortholog database) and GO databases. The E-value thresholds were set to 10^{-5}, 10^{-5}, 0.01, 10^{-3}, 10^{-5}, 10^{-10} and 10^{-6}, respectively.

Differential expression analysis and gene classification

For the gene expression analysis, the read counts from each sequenced library were modified using the edgeR program package with a scaling normalised factor. Genes showing differential expression analysis of two samples were identified using the DEGseq-R package. The q value was used to adjust the P value; for significant differential expression, the threshold value was set as $q < 0.005$, and a |log2 (fold change)| > 1 (Storey and Tibshirani 2003).

The GOSeq package was used for enrichment analysis of the DEGs based on the Wallenius non-central hypergeometric distribution, which can adjust for gene length bias (Young et al. 2010). KEGG enrichment analysis of the DEGs was implemented using KOBAS software to understanding the high level functions and biological systems (Mao et al. 2005; Kanehisa et al. 2008).

Real-time RT-PCR analysis

To validate the reliability of gene expression profiling via RNA-Seq and the unigenes obtained from the assembled transcriptomes, the expression levels of 15 selected unigenes related to flavonoid, theanine and caffeine synthesis as well as N transport were identified by real-time RT-PCR. Total RNA was purified with an RNA purification kit (Tiangen, China). cDNA was synthesised using the PrimeScript II 1st Strand cDNA Synthesis Kit from TaKaRa (Dalian, China). Expression patterns of the selected unigenes were monitored and the detailed information on these unigenes is shown in Table 3. The expression levels of unigenes were calculated using the $2^{\Delta Ct}$ method and normalised to that of the actin gene (Livaka and Schmittgen 2001). Three replicates were completed for each reaction, and all data are shown as the mean ± SD after normalisation.

Results

Sequencing, de novo assembly and sequence analysis

To obtain a general overview of the tea plants transcriptomes, the total RNAs from buds, mature leaves, shoots and roots of tea plants treated with NH_4^+, NO_3^- or NH_4^++NO_3^- for 5 min or 96 h were obtained for generate the cDNA library. Illumina RNA-Seq technology was used to sequence the tea plant transcriptomes. After quality checks, including the removal of adaptor sequences containing N reads and low-quality reads, the eight RNA libraries generated a total of 52.65 Gb of clean reads (Table 1), with an average of 6.58 Gb of clean reads per sample.

All clean reads were assembled de novo using the Trinity programme (Grabherr et al. 2015). As a result, a total of 277,768 unigenes (140.38 Mb) were generated, with a mean length of 505 bp. The lengths of unigenes ranged from 201 to 15,244 bp, and the N50 length was 641 bp (Table 1). The size distribution is presented in Fig. 1. In total, 66,035 unigenes (23.77%) were longer than 500 bp, and 27,163 unigenes (9.78%) were longer than 1 kb.

RSEM was used to assemble the reads from eight tea plant samples (Li et al. 2011, Li and Dewey 2011). Using a fragments per kilobase per million reads (FPKM)

Table 1 Summary of transcriptome data

Items	Amounts
Raw sequencing reads	361,418,568
Clean reads	350,931,922
Number of transcripts	374,198
Number of unigenes	277,768
Total nucleotides of transcripts (bp)	263,812,896
Total nucleotides of unigenes (bp)	140,380,222
Max length of unigenes (bp)	15,244
Min length of unigenes (bp)	201
Mean length of unigenes (bp)	505
N50 of unigenes (bp)	641

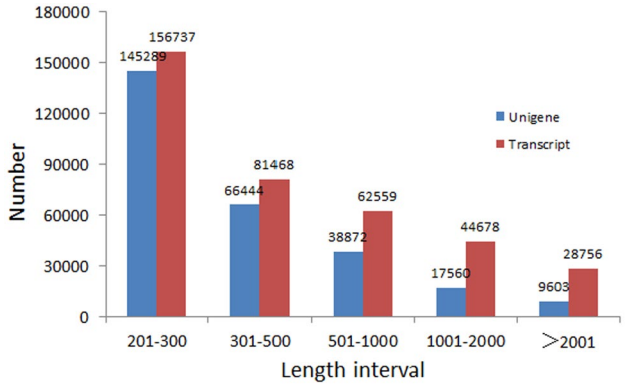

Fig. 1 Length distributions of assembled unigenes and transcripts

Table 2 Summary of annotations of the *C. sinensis* unigenes

	Number of unigenes	Percentage (%)
Annotated in NR	100,453	36.16
Annotated in NT	54,251	19.53
Annotated in KO	52,874	19.03
Annotated in SwissProt	88,327	31.79
Annotated in PFAM	91,578	32.96
Annotated in GO	94,351	33.96
Annotated in KOG	58,665	21.12
Annotated in all databases	17,190	6.18
Annotated in at least one database	139,046	50.05
Total unigenes	277,768	100

value ≥ 0.3, the levels of the unigenes of in each sample were measured (Trapnell et al. 2010). The gene expression distribution of each sample and the dispersion of expression levels are shown in Fig. 2a, b, respectively.

Functional annotation and characterisation of the unigenes

Functional annotation of the 277,768 unigenes revealed 100,453, 54,251, 88,327, 91,578, 52,874, 94,351 and 58,665 unigenes with alignments to the NR, NT, SwissProt, PFAM, KEGG, GO and KOG databases, respectively (Table 2). In all of the databases, 139,046 unigenes could be annotated to at least one database. However, 138,722 (49.95%) of the unigenes did not having matching sequences to any databases mentioned above because of a lack of *C. sinensis* genome information.

A BLAST search of the NR database showed that 36.14% of the unigenes were significant BLAST hits. The E values of 22.8% of the mapped sequences were lower than 1.0 e−60 according to the E-value distribution of the top hits in the NR database. The similarity distribution results suggest that more than 80% of the sequences shared sequence similarities of more than 60%. Homologous genes were found in more than seven species; 12% of the unigenes shared the highest homology with genes from *Vitis vinifera*, followed by *Hordeum vulgare* (4.1%).

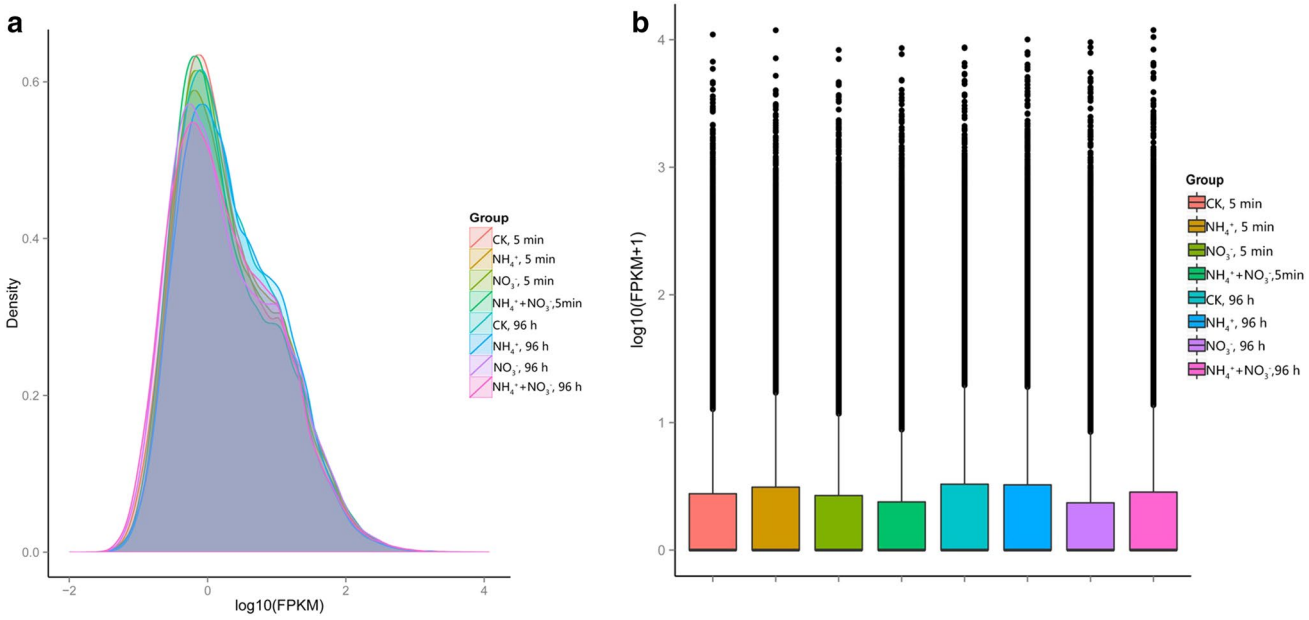

Fig. 2 Levels of expressed unigenes from *C. sinensis* with different treatments. The densities (**a**) and distributions (**b**) of unigenes with different expression levels in each sample

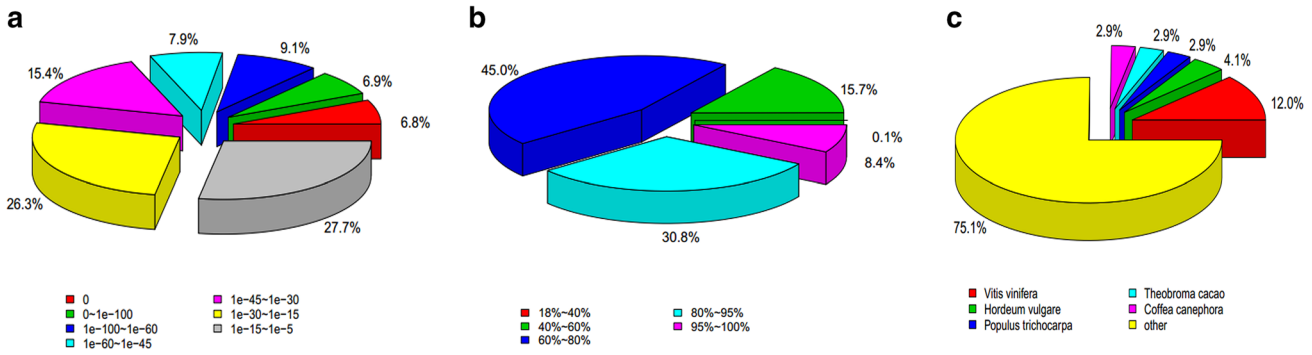

Fig. 3 Characteristics of homology search of unigenes against the NR database. **a** E-value distribution of the top BLAST hits for each unigene (E value of 1.0e−5). **b** Similarity distribution of the best BLAST hits for each unigene. **c** Species distribution is shown as the percentage of the total homologous sequences (with an E value ≤ 1.0e−5)

More than 75% of the unigenes did not share high homology with genes from specific species (Fig. 3).

In total, 58,665 unigenes mapped to 26 KOG clusters (Fig. 4; Supplementary Table 1). 64,644 functional annotations were produced, as some unigenes were annotated with multiple KOG functions. The five largest categories included the following: (1) translation, ribosomal structure and biogenesis (18.36%); (2) posttranslational modification, protein turnover and chaperones (13.04%); (3) general function prediction only (11.28%); (4) signal transduction mechanisms (7.43%); and (5) energy production and conversion (7.05%). Unigenes involved in secondary metabolite biosynthesis, transport and catabolism represented 2.47% (1594 unigenes) of all KOG-annotated unigenes.

Using GO functional enrichment analysis, 94,351 unigenes were assigned to three categories and 56 functional groups, including cellular components, biological processes and molecular function (Fig. 5; Supplementary Table 2). In the KEGG database, 52,874 unigenes were annotated and mapped to 32 KEGG pathways (Fig. 6; Supplementary Table 3).

Differentially expressed gene (DEG) analysis

DESeq (Anders and Huber 2010) identified 1052 DEGs from the read count values of unigenes from the plant samples treated with NH_4^+, NO_3^- and NH_4^++NO_3^- (Figs. 7, 8). To validate the RNA-Seq results, we selected 15 genes, that

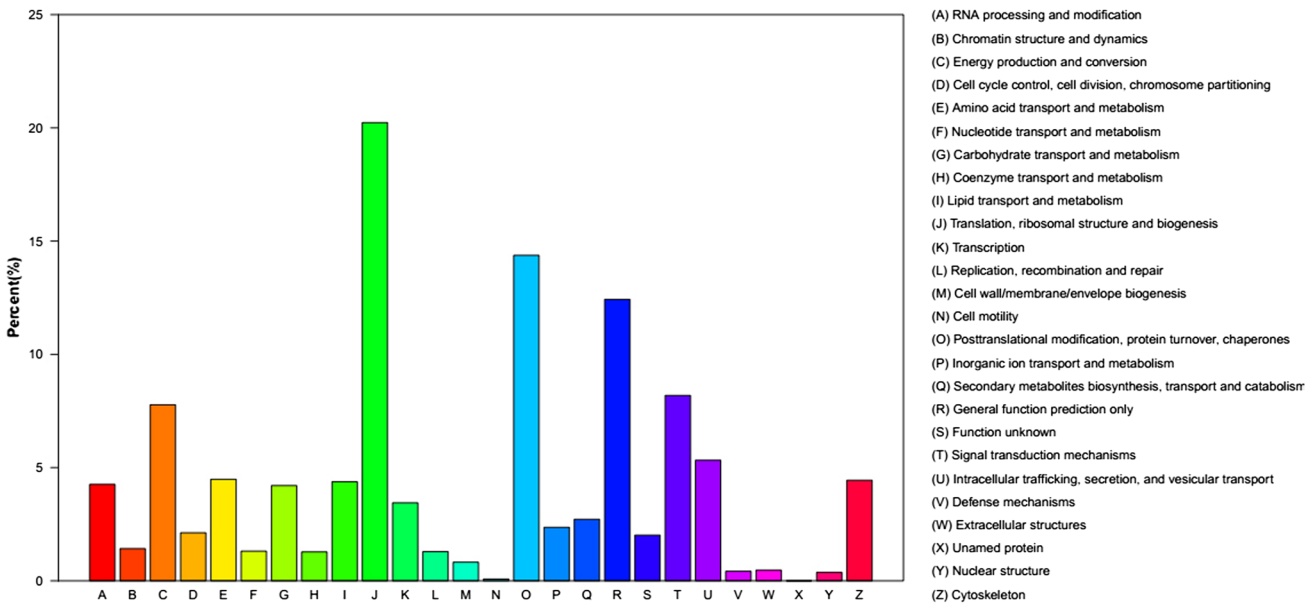

Fig. 4 KOG Classification of the transcriptome in *C. sinensis* treated by different N. A total of 64,644 unigenes showing significant homology to the KOGs database at NCBI (E value ≤ 1.0e−5) have a KOG classification among the 26 categories

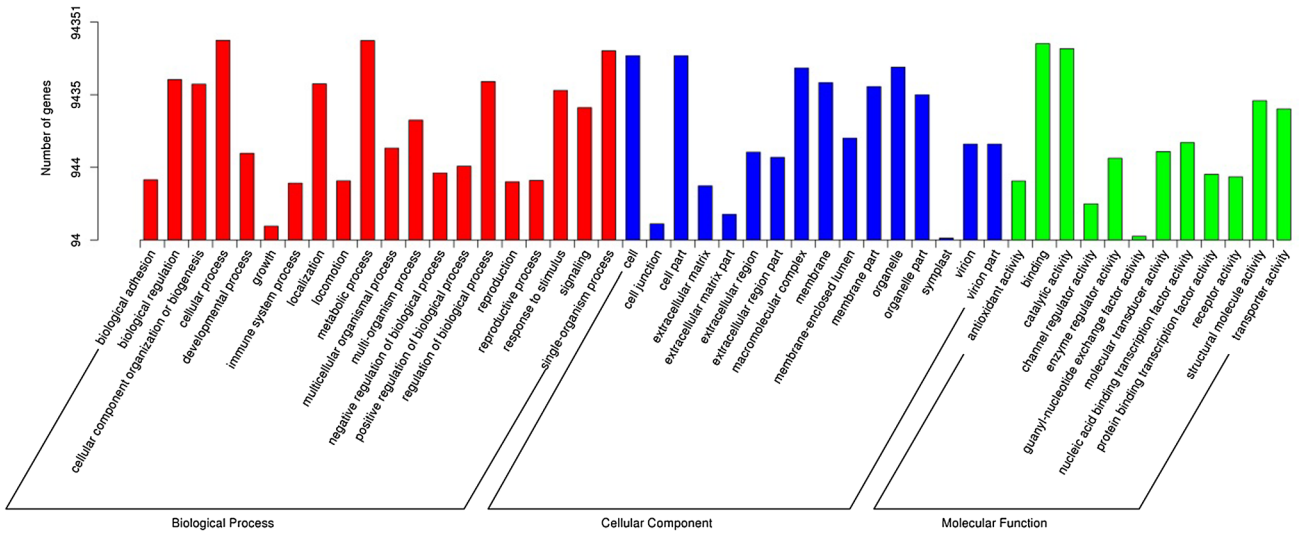

Fig. 5 GO Classification of the transcriptome in *C. sinensis* treated by different N.A total of 94,351 unigenes were assigned to 56 GO pathways in Gene Ontology (E value ≤ 1.0e−6)

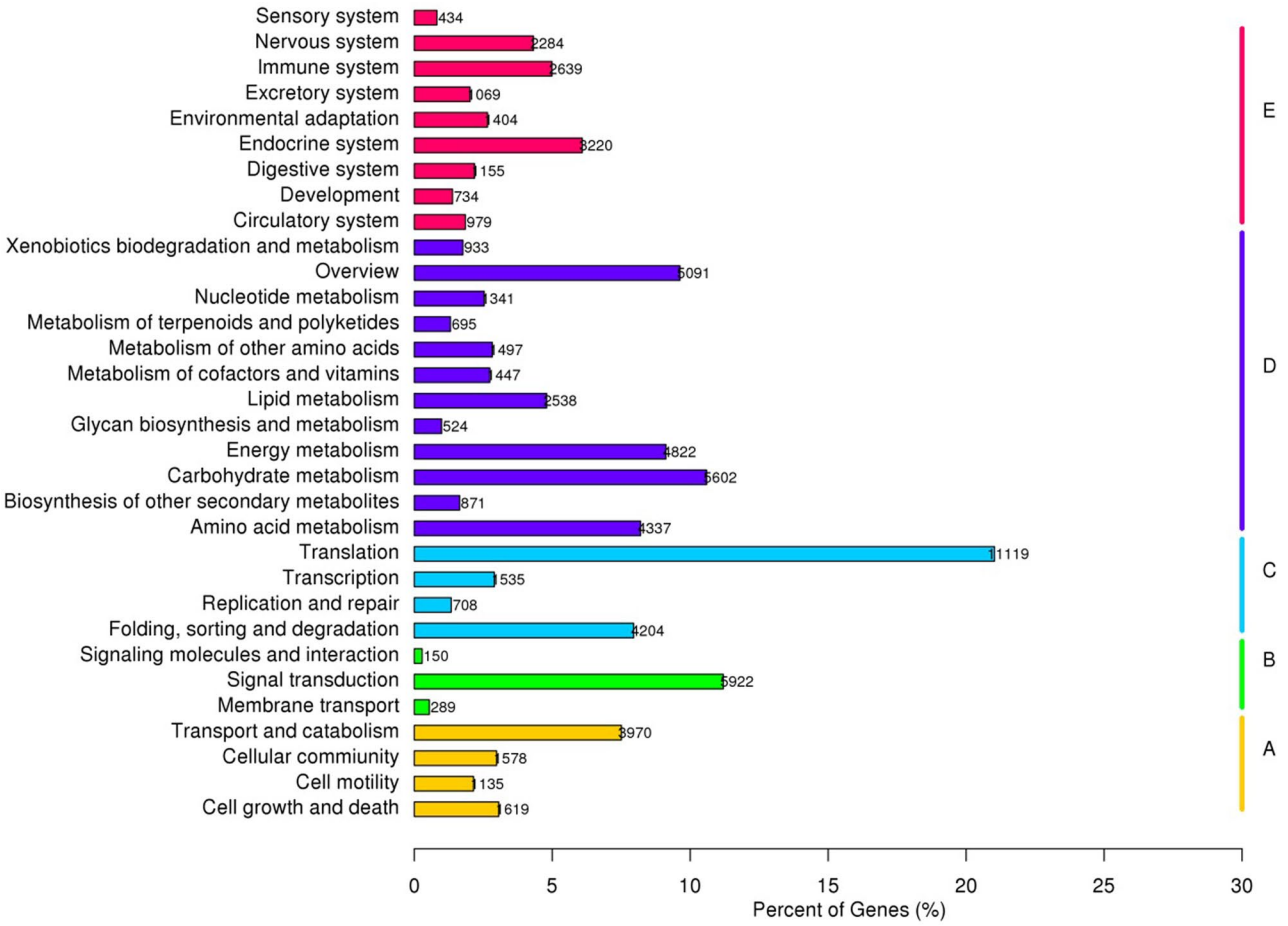

Fig. 6 KEGG Classification of the transcriptome in *C. sinensis* treated by different N.A total of 52874 unigenes were assigned to 32 KEGG pathways in KEGG database (E value ≤ 1.0e−10)

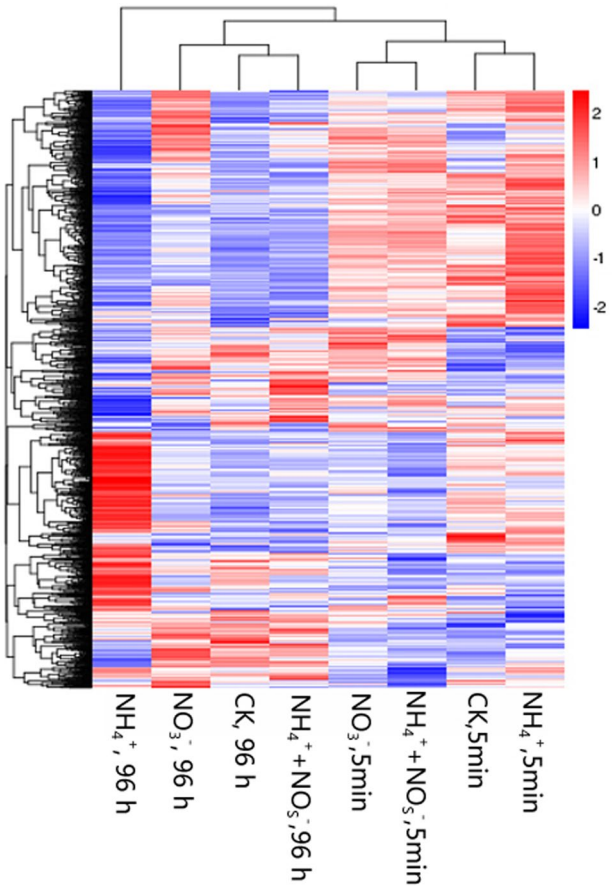

Fig. 7 Cluster analysis of DEGs in tea plants treated by different N treatment. NH_4^+, $NO_3^-$and NH_4^++NO_3^- represent NH_4^+-treatment, NO_3^--treatment and NH_4^++NO_3^--treatment, respectively. 5 min and 96 h represent different treatment time. The signal ratios were shown in a red–green colour scale, where red indicated up-regulation and green indicated down-regulation. The scale represents the logarithms of the RPKM (reads per kilobase per million reads) values of the unigenes

representing a wide range of relative expression levels and different functions and subjected them to RT-PCR analysis (Table 3). The resulting gene expression profiles were consistent with the RNA-Seq data.

Functional annotation by the NR, NT, Swiss-Prot, PFAM, KEGG, GO and COG databases indicated that 175 DEGs were associated with N secondary metabolism and transport (Tables 4, 5, 6, 7; Supplementary Figs. 1, 2, 3, Supplementary Table 4).

Analysis of genes related to secondary metabolite biosynthesis in tea plants fed with different N forms

After 5 min of constant treatment with N, the expression levels of 193 DEGs were up-regulated in all libraries, whereas 87 were down-regulated (Fig. 8). Forty DEGs in tea plants were up-regulated specifically by NH_4^+ treatment, including six flavonoid biosynthesis-related genes (Table 4). In tea plants treated with NO_3^- for 5 min, two flavonoid biosynthesis-related genes and two theanine biosynthesis-related genes were specifically up-regulated, and one flavonoid biosynthesis-related gene and one theanine biosynthesis-related gene were specifically down-regulated (Table 4). Two up-regulated genes, related to flavonoid and caffeine biosynthesis, and six down-regulated genes, including four genes related to the flavonoid biosynthetic pathway, one gene involved in theanine biosynthesis and one gene involved in caffeine biosynthesis, were specifically expressed in tea plants that were treated for 5 min with NH_4^++NO_3^-. Only two genes (c81491_g1 and c92007_g2) involved in theanine biosynthesis in tea plants were up-regulated by all three N treatments (Table 4). These results are in accordance with previous studies (Li and Silva 2011; Li et al. 2015; Ruan et al. 2007b), suggesting that N forms may regulate the biosynthesis of secondary metabolites in tea plants via different pathways. In addition, in both NO_3^- and NH_4^++NO_3^- treatments, five genes associated with the flavonoid biosynthesis pathway and two genes involved in theanine biosynthesis were up-regulated, and one gene involved in flavonoid biosynthesis was down-regulated, suggesting that during the early stage of N treatment, NO_3^- and NH_4^++NO_3^- may share partial pathways to regulate the biosynthesis of secondary metabolites in tea plants.

After 96 h of treatment with different N forms, 456 DEGs were up-regulated and 138 DEGs were down-regulated in all samples (Fig. 8). Fourteen genes involved in flavonoid biosynthesis, 14 genes related to theanine biosynthesis and 1 gene involved in caffeine biosynthesis were up-regulated specifically by NH_4^+. At the same time, eight genes, two genes and two genes involved in flavonoid biosynthesis, theanine biosynthesis and caffeine biosynthesis, respectively, were down-regulated specifically by NH_4^+. However, five genes associated with flavonoid biosynthesis and six genes involved in theanine biosynthesis were up-regulated specifically by NO_3^-, and no genes associated with secondary metabolite biosynthesis were down-regulated (Table 5). In a previous study, after 24 h of constant treatment with NH_4^+, although the N content in the tea roots accumulated, the levels of amino acids in the plants continuously decreased, whereas the amino acid concentrations in plants treated with NO_3^- continuously increased (Yang et al. 2013). This result is in accordance with our study, which found the down-regulation of 12 genes related to secondary metabolism, suggesting that the regulation of secondary metabolite biosynthesis by NH_4^+ in tea plants may be affected by other pathways.

Only three genes encoding proteins in flavonoid biosynthesis were specifically up-regulated by NH_4^++NO_3^-. In addition, our results showed that there were no DEGs found in any NH_4^++NO_3^- treatments associated with theanine

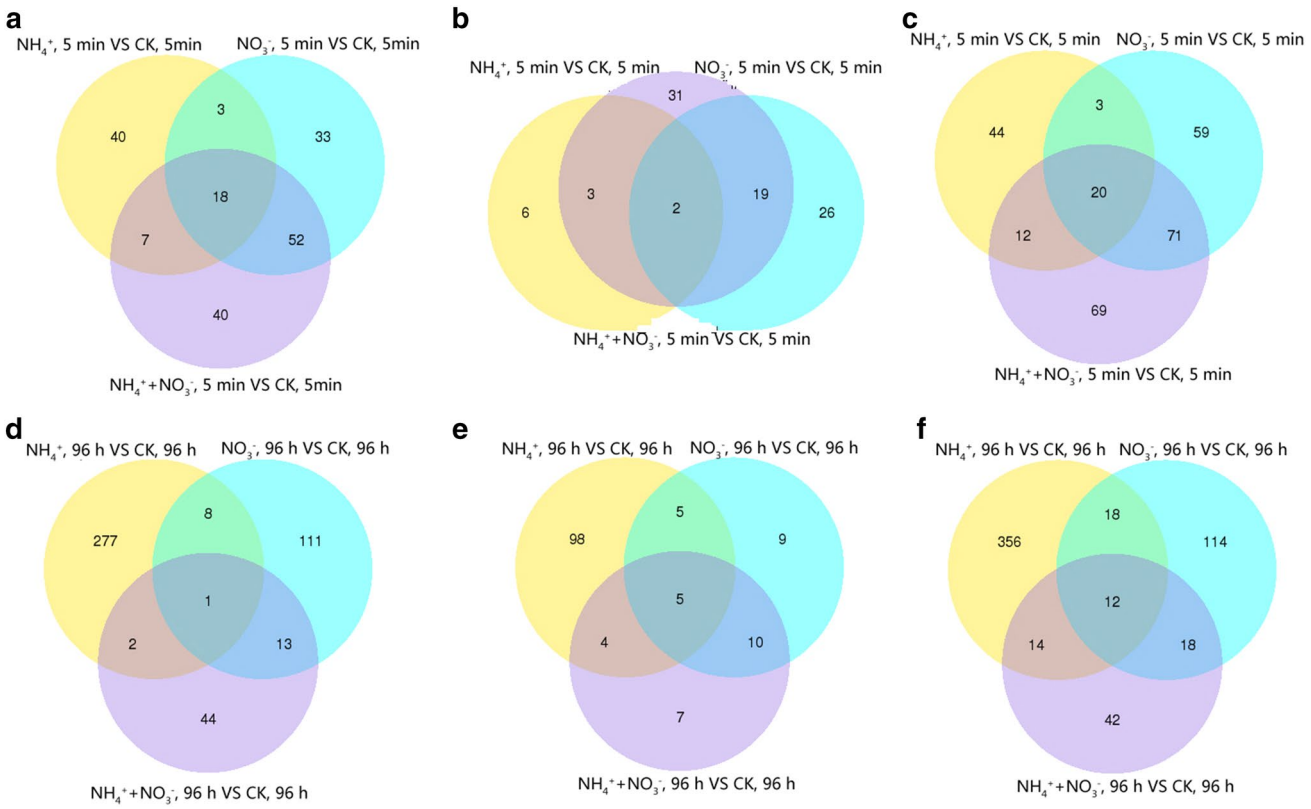

Fig. 8 Venn diagram of co-expressed and uniquely expressed DEGs from *C. sinensis* treated by different N for different treatment time. **a–c** Respectively, shows the up-regulated, down-regulated and all DEGs in plants treated by NH_4^+, NO_3^- and NH_4^++NO_3^- for 5 min. **d–f** The up-regulated, down-regulated and all DEGs in plants treated by NH_4^+, NO_3^- and NH_4^++NO_3^- for 96 h

and caffeine biosynthesis (Table 5), suggesting that persistent NH_4^++NO_3^- might result in a decline in the secondary metabolites of tea plants.

Remarkably, at both 5 min and 96 h after N treatment, the DEGs involved in secondary metabolite biosynthesis in tea plants treated with NH_4^+ were more abundant than those in the plants treated with NO_3^- and NH_4^++NO_3^--fed tea plants (Tables 4, 5), providing evidence of the NH_4^+-preference of tea plants at the gene level.

Effects of N treatment time on genes related to secondary metabolite biosynthesis

To clarify the effects of treatment time on secondary metabolite biosynthesis, the transcriptomic profiles of tea plants fed with N for 5 min were compared with those treated for 96 h. From Supplementary Figs. 1, 2 and 3, in the NH_4^+, NO_3^- and NH_4^++NO_3^- treatment conditions, the expression levels of 463, 274 and 202 DEGs were regulated, respectively.

With NH_4^+ treatment for 5 min, five genes involved in flavonoid biosynthesis and two genes involved in theanine biosynthesis were up-regulated specifically, and no genes related to metabolism biosynthesis were down-regulated. In the NH_4^+ treatment for 96 h, 13 genes involved in flavonoid biosynthesis, 6 genes involved in theanine biosynthesis and 2 genes related to caffeine biosynthesis were up-regulated specifically, and 8 genes involved in flavonoid biosynthesis, 2 genes related to theanine biosynthesis and 2 genes related to caffeine biosynthesis were down-regulated specifically (Supplementary Table 4). These results showed that in NH_4^+ treatment conditions, the increase of treatment time resulted in simultaneous increases in the numbers of both up-regulated and down-regulated genes involved in secondary metabolism, suggesting that the activations of some secondary metabolic pathways might be time dependent and that they may not be activated in the early stage of NH_4^+ treatment. With increasing treatment time, the biosynthesis of secondary metabolites of tea plants fed with NH_4^+ may become enhanced. However, the numbers of down-regulated genes involved in secondary metabolism, including several genes involved in amino acid metabolism (such as c92029_g2, c91124_g4, c91124_g2, c92401_g1, c91883_g1 and c94808_g2) (Supplementary Table 4), increased, suggesting the possible negative effects of excess N on tea quality. The present results were closely associated with those of Yang

Table 3 Verification of the relative expression levels of DEGs by real-time PCR

Gene ID	Treatment	Oligonucleotide primers	Gene function	log_2 fold change	
				RNA-seq	Real-time PCR
c92887_g2	NH_4^+ (5 min)	GTATGGTAACAAGCAGGTC GATGGATAGGCAGATGAA	Gibberellin induced protein	− 2.630	− 1.152
c87347_g1	NH_4^+ (5 min)	TTGGGCATCTGCTGGTTCA CCA CTTGGCGGCATCTCA	NRT1/PTR FAMILY 7.3-like	2.817	2.147
	NO_3^- (5 min)			2.466	0.275
	NO_3^- (96 h)			2.564	3.380
c86792_g1	NO_3^- (5 min)	GGAGATTGTGATTCCCGTA TAGCCATTCTTGTTGTTCG	PREDICTED: pyrophosphate-energised vacuolar membrane proton pump-like	1.802	1.070
c86354_g1	NO_3^- (5 min)	CTTCGGTGCTTTCTACTGT CTCTTATCCCATCCTTCC	Secoisolariciresinol dehydrogenase-like	1.319	2.254
c94647_g1	NO_3^- (5 min)	TTGCCGATGAATGAACCT CTC ACCAGAGCCAAATGC	PREDICTED: 4-hydroxyphenylpyruvate dioxygenase-like	1.321	1.585
c89007_g5	NO_3^- (5 min)	GGGTTCCAAATGGTGTT CTGTGCCAAGGCTATGA	Glutamine synthetase	-1.044	-0.889
c92627_g6	NO_3^- (5 min)	CTGGTCCCTTCATTTCCT CGC CCTGATTTGAGTAGA	Allene oxide synthase	5.253	0.485
	NO_3^- (96 h)			6.714	5.847
c95022_g1	NO_3^- (5 min)	CTTCTGGGTTTGGCTCTA AGT TTCGGCAAGTTATCC	Nodulin MtN21/EamA-like transporter family protein	2.427	1.245
	NO_3^- (96 h)			2.277	2.144
c80549_g1	NO_3^- (96 h)	ACTATCGTCAGGAGGGTG GAG CCAAGGATGATTACAG	PREDICTED: vacuolar amino acid transporter 1-like	2.294	2.606
c83774_g1	NO_3^- (96 h)	GTAGACGGGCTTTGAATC CCATCGGTATCTGGAACA	Zinc-finger protein 1	1.927	0.485
c82528_g1	$NH_4^+ + NO_3^-$ (5 min)	GGCAGCAACGATGAATG AGAACTCGGGTGCAGAA	MYB transcription factor	2.678	0.632
c87669_g1	$NH_4^+ + NO_3^-$ (5 min)	AAGCGAACCACCGAACA TGGAACGACGAAATCTGC	Anthocyanin 5-aromatic acyltransferase	1.539	2.488
c91316_g3	$NH_4^+ + NO_3^-$ (5 min)	ATCTGGCGTTTCCTTGCG TTGCGTGGATGGACTTGC	Flavonoid 3′,5′-hydroxylase	− 1.089	− 1.152
c40800_g1	$NH_4^+ + NO_3^-$ (96 h)	AGGACCTTGGCTACTCTGG CCTTCATCGTCGCATTTC	Choline dehydrogenase	5.544	5.137
c95492_g2	$NH_4^+ + NO_3^-$ (96 h)	CACCTTCCCATTGTTCG TTTGGTGAGCCCAGAAC	Cytochrome P450 isoform 1	1.452	0.911
Actin		AATGGTGAAGGCTGGGTT TGCTTTAGGGTTGAGTGG			

NH_4^+ and NO_3^-represent different N scoures. 5 min and 96 h represent the treatment time

et al. (2013), who showed that the free amino acid contents in tea plants with NH_4^+ increased after 12 h, indicating the importance of effective N utilisation.

After NO_3^- treatment for 5 min, eight genes related to flavonoid biosynthesis were specifically up-regulated, and two genes involved in theanine biosynthesis and one gene associated with caffeine biosynthesis were down-regulated. After 96 h of treatment with NO_3^-, three up-regulated genes involved in flavonoid biosynthesis decreased in the tea plants, whereas three genes involved in theanine biosynthesis and one gene involved in caffeine biosynthesis were up-regulated (Supplementary Table 5). These findings suggested that the increase of treatment time could attenuate the flavonoid biosynthesis and enhance the theanine and caffeine biosynthesis in NO_3^--treated tea plants.

It is interesting that the trend of genes related to secondary metabolite biosynthesis in the NH_4^++NO_3^- treatment was opposite that of the trend in the NH_4^+ treatment (Supplementary Table 6). In the NH_4^++NO_3^- treatment, the numbers of up-regulated and down-regulated genes fell to 3 and 0 from 11 and 6, respectively, accompanied by the increase in treatment time from 5 min to 96 h. We speculated that some regulation mechanism of NH_4^++NO_3^- in tea plants may be unknown.

Moreover, at both 5 min and at 96 h after continually N treatment, only seven DEGs associated with the three types of secondary metabolism were regulated in NH_4^+-treated tea plants, five were regulated in NO_3^--treated tea plants and zero were regulated in NH_4^++NO_3^--treated tea plants

Table 4 DEGs involved in secondary metabolites biosynthesis identified in tea plants fed by N for 5 min

Treatments	Gene ID	Description	KEGG pathway	Metabolic pathway
NH_4^+, NO_3^- and NH_4^++NO_3^-				
Up-regulated	c81491_g1	Asparagine synthetase family protein	Alanine, aspartate and glutamate metabolism	Theanine biosynthesis
	c92007_g2	Chitinase	Amino sugar and nucleotide sugar metabolism	
NH_4^+				
Up-regulated	c92988_g1	Tryptophan synthase beta chain 2		Flavonoid biosynthesis
	c95192_g1	Flavonol synthase		
	c91871_g2	UDP-glycosyltransferase 73D1		
	c86561_g1	Mixed amyrin synthase		
	c88626_g3	*S*-Adenosylmethionine synthase 4; methionine adenosyltransferase	Cysteine and methionine metabolism	
	c90920_g3	*S*-Adenosylmethionine synthase 4; methionine adenosyltransferase	Cysteine and methionine metabolism	
NO_3^-				
Up-regulated	c84197_g1	Putative beta-glucosidase 41-like	Phenylpropanoid biosynthesis; alanine, aspartate and glutamate metabolism; cyanoamino acid metabolism	Flavonoid biosynthesis
	c86354_g1	Secoisolariciresinol dehydrogenase-like	Carotenoid biosynthesis	
	c77862_g2	JCGZ_17723	Beta-Alanine metabolism, Tryptophanmetabolism, ARGININE and proline metabolism	Theanine biosynthesis
	c90436_g2	Aldehyde dehydrogenase family 2 member B7	Limonene and pinenedegradation, histidine metabolism	
Down-regulated	c86718_g3	Phosphoenolpyruvate carboxylase 4	Pyruvate metabolism	Flavonoid biosynthesis
	c89007_g5	Glutamine synthetase		Theanine biosynthesis
NH_4^++NO_3^-				
Up-regulated	c87669_g1	Anthocyanin 5-aromatic acyltransferase		Flavonoid biosynthesis
	c93900_g5	Allantoatedeiminase	Purine metabolism	Caffeine biosynthesis
Down-regulated	c91993_g1	Beta-glucosidase 47 isoform 1	Phenylpropanoid biosynthesis, Cyanoamino acid metabolism	Flavonoid biosynthetic pathway
	c84026_g2	Anthocyanidin reductase	Flavonoid biosynthesis, Carotenoid biosynthesis	
	c91316_g3	Flavonoid 3′,5′-hydroxylase	Flavonoid biosynthesis, Flavone and flavonol biosynthesis	
	c90405_g1	Carotenoid cleavage dioxygenase 4	Carotenoid biosynthesis	
	c85152_g1	Protein ASPARTIC PROTEASE IN GUARD CELL 2		Theanine biosynthetic pathway
	c84429_g4	Methylthioribulose-1-phosphate dehydratase		Caffeine biosynthesis

(Supplementary Tables 4, 5, 6). These results suggest that the early response patterns of tea plants to NH_4^+ or NH_4^++NO_3^- treatment are different from the subsequent response patterns from treatment for 96 h and that N may regulate secondary metabolism through different pathways in different stages of treatments. The increase of treatment time not only changed the number of DEGs but also the pathways of the secondary metabolite biosynthesis.

Effects of N form on DEGs related to N transport and metabolism

Using a scanning ion-selective electrode, the influx rates of NO_3^- were found to be much lower than those of NH_4^+ in tea plant roots treated with NH_4^+, NO_3^- or NH_4^++NO_3^- (Ruan et al. 2016). To elucidate the molecular basis of NH_4^+ preference, the DEGs related to N transport in tea plants fed with NH_4^+, NO_3^- or mixed N forms were analysed (Table 6).

Table 4 (continued)

Treatments	Gene ID	Description	KEGG pathway	Metabolic pathway
NO_3^- and NH_4^++NO_3^-				
Up-regulated	c88932_g5	Arogenate dehydrogenase 1	Phenylalanine, tyrosine and tryptophan biosynthesis	Flavonoid biosynthesis
	c89647_g1	Lysosomal beta-glucosidase-like	Phenylpropanoid biosynthesis, cyanoamino acid metabolism	
	c92029_g2	Hypothetical protein POPTR_0011s11570g	Phenylalanine metabolism; Phenylalanine, tyrosine and tryptophan biosynthesis; cysteine and methionine metabolism	
	c94647_g1	4-Hydroxyphenylpyruvate dioxygenase-like	Phenylalanine metabolism; tyrosine metabolism	
	c90706_g2	Polyphenoloxidase		
	c84529_g2	L-Arabinokinase-like	Amino sugar and nucleotide sugar metabolism	Theanine biosynthesis
	c89430_g6	Tyrosine/alanine aminotransferase-like		
Down-regulated	c94311_g1	Protein SRG1	Flavonoid biosynthetic process	Flavonoid biosynthesis

A q value < 0.005 and $|\log_2$ (fold change)$| > 1$ were set as the threshold for significant differential expression

Altogether, 50 DEGs related to transport were identified in tea plants treated with N. Regarding the 5-min treatment with N, the numbers of DEGs in all three treatments were low, whereas after 96 h of treatment, the quantities of up-regulated DEGs in the NH_4^+ treatment (17) were increased to a much greater extent than those in the NO_3^- (8) and NH_4^++NO_3^- treatments (2). These data are closely associated with the results of Ruan et al. (2016), suggesting that the NH_4^+ preference of tea plants could be attributed to the up-regulation of transport-related DEGs.

To determine the reason for the up-regulation of more transport-related DEGs in the NH_4^+ treatment, the DEGs related to N metabolism were studied. In addition to the genes encoding glutamate synthase and glutamate dehydrogenase involved in secondary metabolism, just two DEGs (c94866_g1 and c89643_g3) involved in N metabolism were identified in the NH_4^+ treatment, and no genes related to N metabolism were found in either the NO_3^- or the NH_4^++NO_3^- treatment. c94866_g1 and c89643_g3 were up-regulated by NH_4^+ treatment for 5 min and 96 h, respectively (Supplementary Table 7). c94866_g1 encodes a bifunctional monodehydroascorbate reductase and carbonic anhydrase nectarin-3-like protein, catalysing the reversible hydration of gaseous CO_2 to carbonic acid, which dissociates to give hydrogen carbonate an above-neutral pH, which functions in arginine and glutamate biosynthesis and metabolism (Fasseas et al. 2011; Leterrier and Río 2005). c89643_g3 encodes a ferredoxin-nitrite reductase, acting with other nitrogenous compounds as donors, with an iron–sulphur protein as an acceptor and participates in nitrogen metabolism and nitrogen assimilation (Hanke et al. 2004). Thus, the two genes might act as key molecule signals that regulate the NH_4^+ metabolism of tea plants.

Specific expression of transcription factors induced by N treatment

Transcription factors are essential for plant growth and developmental processes. Some important transcription factors that play key regulatory roles in tea plant secondary metabolism have been reported (Paul et al. 2014; Li et al. 2015). In this study, we identified 25 transcription factors from ten families were identified in the transcriptomic profiles of tea plants fed with different N forms in this study (Table 7).

Previous studies have demonstrated MYB/bHLH interactions in flavonoid synthesis (Nesi et al. 2001; Baudry et al. 2006). The caffeine biosynthesis pathway is correlated with the bZIP, bHLH and MYB families of transcription factors (Li et al. 2015). GATA and bHLH factors play important roles in caffeine biosynthesis by binding upstream of the transcription start site of 5′-monophosphate deaminase, which is critical in the construction of the xanthene skeleton purine nucleotide pathway (Shi et al. 1997). Theanine biosynthesis is highly correlated with the AP2, bHLH, bZIP, C2H2 and WRKY families of transcription factors. These transcription factors may function by regulating theanine synthetase/glutamine synthetase. It has been reported that an NAC transcription factor was linked to all three pathways via cinnamate-4-hydroxylase of the flavonoid pathway and adenosine 5′-monophosphate deaminase of the caffeine pathway and via the arginine decarboxylase and glutamine

Table 5 DEGs involved in secondary metabolites biosynthesis identified in tea plants fed by N for 96 h

Treatments	Gene ID	Description	KEGG pathway	Secondary metabolism
NH_4^+				
Up-regulated	c92813_g1	Peroxidase 64-like isoform 1	Phenylpropanoid biosynthesis	Flavonoid biosynthesis
	c85158_g1	Chalconeisomerase		Flavonoid biosynthesis
	c84384_g1	Chalcone synthase		Flavonoid biosynthesis
	c92121_g1	Terpene synthase	Flavonoid biosynthesis	Flavonoid biosynthesis
	c86291_g1	Terpene synthase	Flavonoid biosynthesis	Flavonoid biosynthesis
	c92121_g1	Terpene synthase	Flavonoid biosynthesis	Flavonoid biosynthesis
	c86291_g1	Terpene synthase	Flavonoid biosynthesis	Flavonoid biosynthesis
	c95192_g1	Flavonol synthase	Flavonoid biosynthesis	Flavonoid biosynthesis
	c74334_g1	Flavone synthase II		Flavonoid biosynthesis
	c90405_g1	Probable carotenoid cleavage dioxygenase 4	Flavonoid biosynthesis	Flavonoid biosynthesis
	c84026_g1	Anthocyanidin reductase 1	Flavonoid biosynthesis	Flavonoid biosynthesis
	c84026_g2	Anthocyanidin reductase	Flavonoid biosynthesis	Flavonoid biosynthesis
	c67549_g1	Leucoanthocyanidin reductase		Flavonoid biosynthesis
	c94102_g1	Cytochrome P450 86B1-like		Flavonoid biosynthesis
	c92446_g2	Aldehyde dehydrogenase family 3 member F1-like	Beta-alanine metabolism, tryptophan metabolism	Theanine biosynthesis
	c92446_g3	Aldehyde dehydrogenase family 3 member F1	Beta-alanine metabolism, tryptophan metabolism	Theanine biosynthesis
	c94605_g1	Aldehyde dehydrogenase (NAD+)	Beta-alanine metabolism, tryptophan metabolism	Theanine biosynthesis
	c84074_g2	Putative glutamine amidotransferase		Theanine biosynthesis
	c90081_g2	S-Adenosyl-L-methionine-dependent methyltransferases superfamily protein isoform 1		Caffeine biosynthesis
Down-regulated	c78091_g1	4-Hydroxycinnamoyl-CoA ligase 3	Phenylpropanoid biosynthesis, phenylalanine metabolism	Flavonoid biosynthesis
	c94647_g1	4-Hydroxyphenylpyruvate dioxygenase-like	Phenylalanine metabolism, tyrosine metabolism	Flavonoid biosynthesis
	c92029_g2	Tyrosine aminotransferase	Phenylalanine metabolism	Flavonoid biosynthesis
	c91124_g4	Phenylalanine ammonia-lyase		Flavonoid biosynthesis
	c91124_g2	Phenylalanine ammonia-lyase 4, partial		Flavonoid biosynthesis
	c92401_g1	Anthocyanin 5-aromatic acyltransferase		Flavonoid biosynthesis
	c93918_g2	Hydroxycinnamoyl-coenzyme A shikimate/quinatehydroxycinnamoyltransferase		Flavonoid biosynthesis
	c94647_g1	4-Hydroxyphenylpyruvate dioxygenase-like		Flavonoid biosynthesis
	c90163_g5	1-Aminocyclopropane-1-carboxylate oxidase	Cysteine and methionine metabolism	Theanine biosynthesis
	c91883_g1	Glutathione S-transferase-like	Glutathione metabolism	Theanine biosynthesis
	c94808_g2	*S*-Adenosyl-L-methionine-dependent methyltransferases superfamily protein		Caffeine biosynthesis
	c93900_g5	Allantoatedeiminase	Purine metabolism	Caffeine biosynthesis
NH_4^+ and NO_3^-				
Up-regulated	c94922_g5	Caffeine synthase		Caffeine biosynthesis

synthetase genes of the theanine pathway (Li et al. 2015). In addition, these transcription factors might mediate the crosstalk between the flavonoid, theanine and caffeine biosynthesis pathways in tea plants.

In tea plants treated with different N forms, we identified three MYB transcription factors (c82528_g1, c88255_g2 and c83332_g1), one GATA transcription factor (c89290_g1), one bHLH transcription factor (c91169_g2),

Table 5 (continued)

Treatments	Gene ID	Description	KEGG pathway	Secondary metabolism
NO_3^-				
Up-regulated	c91134_g1	Bifunctional 3-dehydroquinate dehydratase/shikimate dehydrogenase	Phenylalanine, tyrosine and tryptophan biosynthesis	Flavonoid biosynthesis
	c91361_g3	Xyloglucanendotransglycosylase/hydrolase	Flavonoid biosynthesis, flavone and flavonol biosynthesis	Flavonoid biosynthesis
	c84619_g2	Cytochrome P450 CYP4/CYP19/CYP26 subfamilies	Carotenoid biosynthesis	Flavonoid biosynthesis
	c86354_g1	Secoisolariciresinol dehydrogenase-like (3-hydroxyacyl-CoA dehydrogenase)	Carotenoid biosynthesis	Flavonoid biosynthesis
	c89430_g6	Tyrosine aminotransferase-like		Flavonoid biosynthesis
	c76368_g1	Mitochondrial aldehyde dehydrogenase	Arginine and proline metabolism, beta-alanine metabolism	Theanine biosynthesis
	c77862_g2	Aldehyde dehydrogenase family	Arginine and proline metabolism, beta-ALANINE metabolism	Theanine biosynthesis
	c90436_g2	Aldehyde dehydrogenase family 2 member B7	Beta-alanine metabolism, tryptophan metabolism	Theanine biosynthesis
	c81491_g1	Asparagine synthetase family protein	Alanine, aspartate and glutamate metabolism	Theanine biosynthesis
	c84308_g1	Homocysteine S-methyltransferase 3-like isoform X2	Cysteine and methionine metabolism	Theanine biosynthesis
	c89623_g2	Arginine decarboxylase	Arginine and proline metabolism	Theanine biosynthesis
NH_4^++NO_3^-				
Up-regulated	c40800_g1	Choline dehydrogenase		Flavonoid biosynthesis
	c87045_g3	Flavonol synthase/flavanone 3-hydroxylase-like		Flavonoid biosynthesis
	c95492_g2	Cytochrome P450 isoform 1		Flavonoid biosynthesis

A q value < 0.005 and $|\log_2$ (fold change)$| > 1$ were set as the threshold for significant differential expression

one AP2 transcription factor (c56762_g1), seven Zinc finger transcription factors (c92755_g1, c91627_g1, c87390_g1, c83774_g1, c89271_g1,c95135_g1 and c20352_g1), one WRKY transcription factor (c91610_g1) and four NAC transcription factors (c91610_g1, c88220_g2, c92489_g1 and c78659_g1), suggesting that the regulation of N in secondary metabolite biosynthesis may be mediated by these transcription factors (Table 7). Several transcription factors were found in more than one N treatment, such as the NAC transcription factor c91610_g1 found in plants treated with NH_4^+, NH_4^++NO_3^- or NO_3^- for 5 min and in plants treated with NO_3^- for 96 h, revealing possible key links between the flavonoid, theanine and caffeine biosynthesis pathways regulated by N.

In addition, some differentially expressed transcription factors involved in phytohormone signal transduction were also identified in tea plants fed with N, suggesting that crosstalk may occur between the responses to N and phytohormones in tea plants. This possibility is further confirmed by the nine DEGs involved in plant hormone signal transduction found in tea plants treated with N (Supplementary Table 8).

Discussion

Flavonoids, theanine and other secondary metabolites play important roles in the quality, development and defence responses of tea plants to environmental stimuli. The regulatory network for secondary metabolite biosynthesis is very complicated. As one of the most important nutrients, N has a great impact on secondary metabolism in plants. This study analysed the transcriptomic profiles of tea plants via RNA-Seq and clarified the molecular bases of different regulation patterns of different N forms in secondary metabolite biosynthesis. The results increase our understanding of the effects of N form on secondary metabolic pathways, providing a fundamental basis for genetic selection and improvements in fertilisation management.

Many key genes in flavonoid, caffeine and theanine metabolic pathways in tea plants, such as genes encoding flavonol synthases (c95192_g1, c74334_g1 and c87045_g3), tyrosine amino transferases (c89430_g6 and c92029_g2), anthocyanidin reductases (c84026_g1 and c84026_g2), aldehyde dehydrogenases (c92446_g2, c92446_g3,

Table 6 DEGs related to N transport identified in tea plants fed by N

Treatment	Gene ID	Description
5 min		
Up-regulated		
NH_4^+	c90828_g2	Amino acid permease 2-like
	c90701_g1	Calcium-transporting ATPase 2
	c93875_g1	ABC transporter A family member 2-like
NO_3^-	c86792_g1	Pyrophosphate-energized vacuolar membrane proton pump-like
NH_4^++NO_3^-	c94175_g1	Calcium permeable stress-gated cation channel 1-like
	c81608_g1	Aquaporin protein AQU21
	c93097_g3	Drug transmembrane transporter activity
NO_3^- and NH_4^++NO_3^-	c87960_g2	Nitrate transporter At1g22540-like
Down-regulated		
NO_3^-	c91495_g3	ABC transporter C family member 8-like
NH_4^++NO_3^-	c90516_g1	Translocon at the outer membrane of chloroplasts 64
NO_3^- and NH_4^++NO_3^-	c83566_g1	WAT1-related protein At5g07050-like
	c93182_g1	WAT1-related protein At2g37460-like isoform X1
96 h		
Up-regulated		
NH_4^+	c89649_g2	ABC transporter
	c95187_g1	ABC transporter B family member 2
	c95187_g3	ABC transporter B family member 10
	c82613_g1	ABC transporter family protein
	c57697_g1	ABC transporter B family member 13-like
	c57722_g1	Aquaporin protein 23
	c78403_g1	Aquaporin protein 12
	c210302_g1	GDSL esterase/lipase 7-like
	c91179_g5	GDSL esterase/lipase At3g48460
	c81328_g1	GDSL-motif lipase/hydrolase family protein
	c90141_g2	Hexose transport protein
	c70422_g1	Copper transporter
	c73470_g1	Protein TAP1 precursor-like
	c85981_g1	Transport inhibitor response 1
	c87062_g2	Auxin efflux carrier component
NO_3^-	c85638_g1	ABC transporter B family member 26
	c78773_g1	Major facilitator superfamily protein
	c95022_g1	Nodulin MtN21/EamA-like transporter family protein
	c91734_g1	Aquaporin PIP2.2
	c87347_g1	Protein NRT1/PTR FAMILY 7.3-like
	c87996_g4	Tonoplastdicarboxylate transporter
	c80549_g1	Vacuolar amino acid transporter 1-like
NO_3^- and NH_4^++NO_3^-	c90516_g1	Translocon at the outer membrane of chloroplasts 64
NH_4^+ and NO_3^- and NH_4^++NO_3^-	c86091_g2	GDSL esterase/lipase APG
Down-regulated		
NH_4^+	c92093_g1	ABC transporter G family member 11-like
	c96072_g1	ABCG/PDR subfamily ABC transporter
NH_4^+ and NO_3^- and NH_4^++NO_3^-	c86792_g1	Pyrophosphate-energized vacuolar membrane proton pump-like

A q value < 0.005 and $|\log_2$ (fold change)$| > 1$ were set as the threshold for significant differential expression

Table 7 Differentially expressed transcription factors in tea plants treated by N

Treatment	Gene ID	Description
5 min		
Up-regulated		
NH_4^+	c56762_g1	AP2 domain-containing family protein
	c91627_g1	RING/c3HC4/PHD zinc finger-like protein
	c83231_g1	Chitin-inducible gibberellin-responsive protein 1-like
NH_4^++NO_3^-	c82528_g1	MYB transcription factor
	N2N3 up	
	c88220_g2	Nam-like protein
	c92755_g1	RING finger and CHY zinc finger domain-containing protein 1 isoform 2
NH_4^+ and NO_3^- and NH_4^++NO_3^-	c90482_g1	WRKY transcription factor 40
	c91610_g1	NAC domain class transcription factor isoform 1
	c87347_g1	Ethylene response factor 12
	N1 up	
96 h		
Up-regulated		
NH_4^+	c86685_g2	Auxin-responsive protein IAA
	c87390_g1	RING/FYVE/PHD zinc finger superfamily protein
	c88255_g2	Myb domain protein 7, putative
	c89290_g1	PREDICTED: GATA transcription factor 9-like
	c91169_g2	PREDICTED: transcription factor bHLH87-like
NO_3^-	c78659_g1	NAC transcription factor 027
	c83774_g1	Zinc-finger protein 1
	c89271_g1	Zinc finger CCCH domain-containing protein 29-like
	c91610_g1	NAC domain class transcription factor isoform 1
	c91627_g1	RING/c3HC4/PHD zinc finger-like protein
	c95135_g1	Zinc finger protein ZAT10-like
	c86645_g3	CCR4-NOT transcription complex family protein
	c77265_g1	Ethylene response factor 4
NH_4^++NO_3^-	c79705_g1	Ethylene-responsive transcription factor RAP2-3
Down-regulated		
NH_4^+	c92489_g1	NAC transcription factor
	c92755_g1	RING finger and CHY zinc finger domain-containing protein 1 isoform 2
	c69704_g1	Ethylene response factor 12/2
$NH_4^+$and NO_3^-	c83332_g1	Transcription factor MYB59-like
NO_3^- and NH_4^++NO_3^-	c20352_g1	Common plant regulatory factor 6;Basic leucine-zipper 44;bZIP transcription factor

A q value < 0.005 and $|\log_2$ (fold change)$| > 1$ were set as the threshold for significant differential expression

c94605_g1, c76368_g1, c77862_g2 and c90436_g2), and *S*-adenosyl-L-methionine-dependent methyltransferases (c90081_g2 and c94808_g2), were regulated by different N forms. These genes with the same functions were activated by different N forms, but just a few genes were differentially regulated in more than one treatment, indicating that the pathways of secondary metabolite biosynthesis in tea plants are determined jointly by the N form and treatment.

In our data, the up-regulated DEGs involved in flavonoid biosynthesis in tea plants were more abundant both 5 min and 96 h after being continually treated with NH_4^+ compared with the NO_3^- treatment. However, Fan et al. (2015) reported that the total catechin content in shoot was significantly lower for NH_4^+ treatments than that for NO_3^- treatments and that the levels of catechin biosynthesis- and regulation-related genes, including phenylalanine ammonia-lyase, chalcone isomerase, chalcone synthase and dihydroflavonol 4-reductase genes, were higher in plants supplied with NO_3^-. These inconsistencies may be caused by different treatment times and DEGs, especially considering the differences in DEGs in tea plants treated with N for different times in this study.

Four DEGs related to caffeine biosynthesis were identified in tea plants treated with N for 96 h in our study (Table 5). c90081_g2, which encodes *S*-adenosyl-L-methionine-dependent methyltransferase, was up-regulated in NH_4^+-fed tea plants. c94922_g5, encoding a caffeine synthase, was up-regulated in NH_4^+- and NO_3^--treated plants. c93900_g5, encoding an allantoate deiminase involved in purine metabolism and c94808_g2, encoding *S*-adenosyl-L-methionine-dependent methyltransferase, were down-regulated in tea plants treated with NH_4^+. In a study by Ruan et al. (2007b), the caffeine contents increased in NH_4^+- and NH_4^++NO_3^--treated tea plants, whereas the catechin contents were reduced in NH_4^++NO_3^--treated tea plants. The reasons for the discrepancies between the gene expression levels and the change in contents of the secondary metabolites require further study.

After 5 min of treatment with N, 280 DEGs, including 42 DEGs involved in the synthesis and metabolism of flavonoids, theanine, amino acids and other secondary metabolites, were detected, suggesting that the responses of plants to N treatment are very fast. However, all the studies regarding content variation of secondary metabolites in tea plants analysed only the content variation after 30 min of treatment with N, much longer than a 5-min treatment period (Ruan et al. 2007a, b, 2016; Yang et al. 2013). The expression analyses of genes involved in secondary metabolism were performed without N treatment (Shi et al. 2011; Li et al. 2015). Much less is known about the early responses of tea plants to N treatment. After 96 h of treatment with different N forms, the number of DEGs in tea plants increased to 594, and the number of DEGs involved in secondary metabolism increased to 47. Moreover, most DEGs at 96 h were different from DEGs at 5 min. What occurred in the tea plants fed with N from 5 min to 96 h to cause the change in DEGs will be an interesting area of exploration.

The regulation of N uptake appears to be complex. The kinetics of N influx in tea plants followed a classic biphasic pattern, demonstrating the action of a high transport system and a low-affinity transport system. NH_4^+ and NO_3^- are absorbed into tea plants by distinct transport systems: NH_4^+ has two, and NO_3^- has three (Li et al. 2011; Ruan et al. 2016). Although NH_4^+ and NO_3^- transport in plants has been studied at both physiological and the molecular levels, only a few studies on the uptake and transport of N forms and their mechanisms were reported. In this study, 48 DEGs related to transport and two DEGs related to N metabolism were examined in tea plants treated with both NH_4^+ and NO_3^-, and the number of up-regulated DEGs in the NH_4^+ treatment was twice that in the NO_3^- treatment. Similarly, after 15 days of NH_4^+ treatment, three DEGs related to N uptake (the NH_4^+ transporter, NO_3^- transporter gene and the aquaporin protein genes) and two genes related to N assimilation (the glutamine synthetase and synthetase genes) were also identified as the key genes which expressed with tissue specificity (Li et al. 2017). However, these five genes were not found in our study, probably due to the different treatment times. Thus, further studies on these DEGs are important to understand the effects of N on secondary metabolism and how NH_4^+ and NO_3^- are taken up, transported and regulated in tea plants.

Author contribution statement YY and FW designed the experiments, performed data analysis, interpreted results and drafted the manuscript. YY and QW gathered samples and performed the experiments. JR gave guidance of experimental designs and revised the manuscript. All authors had read and approved the final manuscript.

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References

Anders S, Huber W (2010) Differential expression analysis for sequence count data. Genome Biol 11:R106

Baudry A, Caboche M, Lepiniec L (2006) TT8 controls its own expression in a feedback regulation involving TTG1 and homologous MYB and bHLH factors, allowing a strong and cell-specific accumulation of flavonoids in *Arabidopsis thaliana*. Plant J 46:768–779

Chen Z, Chen L (2012) Delicious and healthy tea: an overview. In: Global tea breeding, advanced topics in science and technology in China. Zhejiang University and Heidelberg. Springer, Hangzhou, pp 1–1

de Mejia EG, Ramirez-Mares MV, Puangpraphant S (2009) Bioactive components of tea: cancer, inflammation and behavior. Brain Behav Immun 23:721–731

Dixon RA, Pasinetti GM (2010) Flavonoids and isoflavonoids: from plant biology to agriculture and neuroscience. Plant Physiol 154:453–457

Fan K, Fan D, Ding Z, Su Y, Wang X et al (2015) Cs-miR156 is involved in the nitrogen form regulation of catechins accumulation in tea plant (*Camellia sinensis* L.). Plant Physio Biochem 97:350–360

Fasseas MK, Tsikou D, Flemetakis E et al (2011) Molecular and biochemical analysis of the alpha class carbonic anhydrases in Caenorhabditis elegans. Mol Biol Rep 38:1777–1785

Grabherr MG, Haas BJ, Yassour M et al (2015) Global transcriptome and gene regulation network for secondary metabolite biosynthesis of tea plant (*Camellia sinensis*). BMC Genom 16:560

Hanke GT, Kimata-Ariga Y, Taniguchi I et al (2004) A post genomic characterization of arabidopsis ferredoxins. Plant Physiol 134:255–264

Hewavitharanage P, Karunaratne S, Kumar NS (1999) Effect of caffeine onshot-hole borer beetle (*Xyleborus fornicatus*) of tea (*Camellia sinensis*). Phytochemistry 51:35–41

Kamau DM, Spiertz JHJ, Oenema O, Owuor PO (2008) Productivity and nitrogen use of tea plantations in relation to age and genotype. Field Crops Res 108:60–70

Kanehisa M, Araki M, Goto S et al (2008) KEGG for linking genomes to life and the environment. Nucleic Acids Res 36:D480–D484

Leterrier M, Río L (2005) Peroxisomal monodehydroascorbate reductase. Genomic clone characterization and functional analysis under environmental stress conditions. Plant Physiol 138:2111–2123

Li B, Dewey C (2011) RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinform 12:323

Li N, Silva J De (2011) Theanine: its occurrence and metabolism in tea. Ann Plant Rev 42:171–206

Li CF, Zhu Y, Yu Y et al (2011) Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat Biotechnol 29:644–652

Li CF, Zhu Y, Yu Y et al (2015) Global transcriptome and gene regulation network for secondary metabolite biosynthesis of tea plant (*Camellia sinensis*). BMC Genom 16:560

Li ZX, Yang WJ, Jala G (2016) Developmental changes in carbon and nitrogen metabolism affect tea quality in different leaf position. Plant Physiol Biochem 106:327–335

Li W, Xiang F, Zhong M et al (2017) Transcriptome and metabolite analysis identifies nitrogen utilization genes in tea plant (*Camellia sinensis*). Sci Rep 7:1693

Livaka KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR. Methods 25:402–408

Mao X, Cai T, Olyarchuk JG, Wei L (2005) Automated genome annotation and pathway identification using the KEGG Orthology (KO) as a controlled vocabulary. Bioinformatics 21:3787–3793

Nesi N, Jond C, Debeaujon I et al (2001) The Arabidopsis TT2gene encodes an R2R3 MYB domain protein that acts as a key determinant for proanthocyanidin accumulation in developing seed. Plant Cell 13:2099–2114

Paul A, Jha A, Bhardwaj S et al (2014) RNA-seq-mediated transcriptome analysis of actively growing and winter dormant shoots identifies non-deciduous habit of evergreen tree tea during winters. Sci Rep 4:5932

Ruan J, Gerendás J, Härdter R, Sattelmacher B (2007a) Effect of nitrogen form and root-zone pH on growth and nitrogen uptake of Tea (*Camellia sinensis*) plants. Ann Bot 99:301–310

Ruan J, Gerendás J, Härdter R, Sattelmacher B (2007b) Effect of root zone pH and form and concentration of nitrogen on accumulation of quality-related components in green tea. J Sci Food Agric 87:1505–1516

Ruan L, Wei K, Wang L et al (2016) Characteristics of NH_4^+ and NO_3^- fluxes in tea (*Camellia sinensis*) roots measured by scanning ion-selective electrode technique. Sci Rep 6:38370

Shi D, Winston JH, Blackburn MR et al (1997) Diverse genetic regulatory motifs required for murine adenosine deaminase gene expression in the placenta. J Biol Chem 272:2334–2341

Shi CY, Yang H, Wei CL et al (2011) Deep sequencing of the *Camellia sinensis* transcriptome revealed candidate genes for major metabolic pathways of tea-specific compounds. BMC Genom 12:131

Storey JD, Tibshirani R (2003) Statistical significance for genome wide studies. Proc Natl Acad Sci USA 100:9440–9445

Trapnell C, Williams BA, Pertea G et al (2010) Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nat Biotechnol 28:511–515

Yamaguchi S, Ninomiya K (2000) Umami and food palatability. J Nutr 130:921–926

Yang YY, Li XH, Ratcliffe RG, Ruan JY (2013) Characterization of ammonium and nitrate uptake and assimilation in roots of tea plants (*Camellia sinensis* (L.) O. Kuntze). Russian J Plant Physiol 60:91–99

Young MD, Wakefield MJ, Smyth GK, Oshlack A (2010) Gene ontology analysis for RNA-seq: accounting for selection bias. Genome Biol 11:1–12