Transcriptome analysis of tea (Camellia sinensis) leaves in response to ammonium starvation and recovery

Yu Wang1, Jia-Xue Ouyang1, Dong-Mei Fan1, Shu-Mao Wang1, Yi-Min Xuan1, Xiao-Chang Wang1,2* and Xin-Qiang Zheng1*

1College of Agriculture and Biotechnology, Tea Research Institute, Zhejiang University, Hangzhou, China, 2Institute of Dafo Longjing, Xinchang, China

The tea plant is a kind of ammonium-preferring crop, but the mechanism whereby ammonium (NH₄⁺) regulate its growth is not well understood. The current study focused on the effects of NH₄⁺ on tea plants. Transcriptomic analysis was performed to investigate the early- and late-stage NH₄⁺ deprivation and resupply in tea plants shoots. Through short- and long-term NH₄⁺ deficiency, the dynamic response to NH₄⁺ stress was investigated. The most significant effects of NH₄⁺ deficiency were found to be on photosynthesis and gene ontology (GO) enrichment varied with the length of NH₄⁺ deprivation. Enriched KEGG pathways were also different when NH₄⁺ was resupplied at different concentrations which may indicate reasons for tolerance of high NH₄⁺ concentration. Using weighted gene co-expression network analysis (WGCNA), modules related to significant tea components, tea polyphenols and free amino acids, were identified. Hence, NH₄⁺ could be regarded as a signaling molecule with the response of catechins shown to be higher than that of amino acids. The current work represents a comprehensive transcriptomic analysis of plant responses to NH₄⁺ and reveals many potential genes regulated by NH₄⁺ in tea plants. Such findings may lead to improvements in nitrogen efficiency of tea plants.

KEYWORDS transcriptionome, ammonium, tea plants, RNA-sequencing, nitrogen, WGCNA

Introduction

Nitrogen is an indispensable nutrient element for plant growth and represents a major driving force for crop yield improvement (Li et al., 2017). Its absorption and assimilation are similarly important for growth of tea plants. In addition to effects on plant growth and development, nitrogen nutrition also affects their ability to cope with environmental challenges (Vega et al., 2015). Nitrogen affects growth of plant roots...
and leaves (von Wiren et al., 2000; Menz et al., 2016; Coletto et al., 2017), senescence rate (Vanacker et al., 2006) and flowering time (Castro Marin et al., 2011). The two main sources of inorganic nitrogen for plants are nitrate (NO$_3^-$) and ammonium (NH$_4^+$) and different plants have different preferences for them. For example, maize and tomato grow better with nitrate, while rice prefers NH$_4^+$ (Britto and Kronzucker, 2002). Previous studies have demonstrated the signaling role of NH$_4^+$ (Ren et al., 2020), which induces a variety of morphological and physiological responses (Liu and von Wirén, 2017). For example, NH$_4^+$ inhibited root growth and affected cell elongation and division in primary roots (Li et al., 2010) but local NH$_4^+$ supply increased lateral root initiation and higher-order lateral root branching (Lima et al., 2010). NH$_4^+$ also promoted the alleviation of toxicity by hormones, such as auxin (Esteban et al., 2016).

Tea is a favored beverage throughout the world. The harvested leaves contain many primary and secondary metabolites, such as catechins, theanine and caffeine. A great deal of research has investigated regulatory mechanisms behind the biosynthesis of metabolites, including the regulation of nutrient content (Ruan et al., 2010). Tea plants have been reported to be well adapted to high NH$_4^+$ concentrations and grow better with a supply of NH$_4^+$ (Fan et al., 2015), a characteristic confirmed by the $^{15}$N study of hydroponic tea plants (Tang et al., 2020). Previous studies found a better nitrogen performance of NH$_4^+$ in tea plants, with increased free amino acids content and reduced secondary metabolites content (Ruan et al., 2007; Yang et al., 2013). NH$_4^+$ benefitted tea plant nitrogen metabolism and upregulated expression of ammonium-assimilation genes (Wang et al., 2021). However, knowledge of effects of NH$_4^+$ on the biosynthesis of tea components and molecular mechanisms involved has received little attention. To this end, Zhang et al. (2018) have established the limiting and sufficient nitrogen conditions for tea growth in a hydroponic system. This current work explored the effects of NH$_4^+$ deficiency and recovery by different ammonium concentrations on physiology and gene regulation in tea plants by the hydroponic method.

Advances in transcriptome analysis technologies have allowed thousands of genes related to nitrogen metabolism in tea plants to be identified. Evaluations of different N sources in tea plants (Liu et al., 2017; Yang et al., 2018; Wang et al., 2021) have found that NH$_4^+$ took precedence over NO$_3^-$ in assimilation. The work of Zhang et al. (2020) investigated the nitrogen uptake in tea roots and identified a group of modules related to low nitrogen treatment using weighted gene co-expression network analysis (WGCNA). Yang et al. (2020) focused on the relationship between nitrogen and amino acid metabolism. The current study explored the effects of NH$_4^+$ deficiency time and NH$_4^+$ concentration on gene expression in tea plants by hydroponics to reveal using RNA-seq coupled with a time-course experiment. Short- and long-term responses of tea plants to NH$_4^+$ deprivation and different concentrations of NH$_4^+$ resupply were assessed and a comprehensive and integrated dataset generated.

**Materials and methods**

**Plant materials and experimental treatments**

One-year-old “Longjing 43” tea seedlings were hydroponically cultivated in distilled water for 7 days and exposed to 1/4 strength nutrient solution for 1 week. The strength of the nutrient solution was thereafter increased to 1/2 (for around 2 weeks) and full (Zhang et al., 2018). The full nutrient solution contained the following nutrients: 1.5 mM (NH$_4$)$_2$SO$_4$, 0.73 mM KNO$_3$, 0.1 mM KH$_2$PO$_4$, 0.46 mM K$_2$SO$_4$, 0.41 mM MgSO$_4$, 0.5 mM CaCl$_2$, 0.046 mM H$_3$BO$_3$, 0.09 mM MnSO$_4$, 0.0091 mM ZnSO$_4$, 0.002 mM CuSO$_4$, 0.0026 mM Na$_2$MoO$_4$, and 0.032 mM Fe-EDTA (Wang et al., 2022). After preculture for 1 month, tea seedlings with a similar appearance were chosen to be transferred to nutrient solution without N for 6 h (S_UN) or 7 days (L_UN) and controls were incubated at a normal NH$_4^+$ level (3 mM NH$_4^+$) for 6 h (CK1) or 7 days (CK2). After 1-week nitrogen deficiency, the nutrient solution was resupplied with 3 mM NH$_4^+$ (NN) or 10 mM NH$_4^+$ (high NH$_4^+$ level, HN) for 6 h (Figure 1A). The pH was adjusted to 5.0 ± 0.2 and solutions were refreshed every 4 days. All hydroponic solutions were continuously aerated using air pumps. Shoots with a bud and two leaves (three biological replicates for each treatment) were harvested, immediately snap-frozen in liquid nitrogen and stored at −80°C for analysis.

**Analysis of tea polyphenols and free amino acids**

Each sample (0.15 g) was extracted with 25 mL of 50% methanol at 70°C for 20 min and centrifuged at 5,000 g for 10 min. The supernatants were collected for further analysis. The concentration of total polyphenols was determined using the Folin-Ciocalteu method. The content of total amino acids was determined by ninhydrin colorimetry according to Shao et al. (2008).

**RNA isolation, cDNA library construction and Illumina deep sequencing**

Total RNA was extracted from the tissue using TRIzol reagent (Plant RNA Purification Reagent for plant tissue, Invitrogen), according to the manufacturer’s instructions
and genomic DNA was removed using DNase I (TaKaRa). RNA quality was determined with a the 2100 Bioanalyzer (Agilent) and quantified by NanoDrop 2000. Only high-quality RNA samples (OD260/280 = 1.8~2.2, OD260/230 ≥ 2.0, RIN ≥ 6.5, 28S:18S ≥ 1.0 > 1 µg) were used to construct the sequencing library.

An RNA-seq transcriptome library was prepared with TruSeq™ RNA sample preparation Kit from Illumina (San Diego, CA) using 1 µg of total RNA. Firstly, messenger RNA was isolated by polyA selection using oligo (dT) beads and then fragmented by fragmentation buffer. Double-stranded cDNA was synthesized using a SuperScript double-stranded cDNA synthesis kit (Invitrogen, CA) with random hexamer primers (Illumina). cDNA was subjected to end-repair, phosphorylation and “A” base addition according to the Illumina library construction protocol. Libraries were size-selected for cDNA target fragments of 300 bp on 2% low range ultra agarose followed by PCR amplification using Phusion DNA polymerase (NEB) for 15 PCR cycles. After quantifying by TBS380, the paired-end RNA-seq sequencing library was sequenced using the Illumina HiSeq XTen/NovaSeq 6000 sequencer (2 × 150 bp read length).

Mapping the RNA-seq reads

The raw paired-end reads were trimmed and quality controlled by using the SeqPrep1 and Sickle software2 with the default parameters. Clean reads were aligned to the tea

1 https://github.com/jstjohn/SeqPrep
2 https://github.com/najoshi/sickle
Differential expression analysis and functional enrichment analysis

In order to identify differentially expressed genes (DEGs) between two different samples, the expression level of each transcript was calculated according to the fragments per kilobase per million reads (FPKM) method. RSEM\(^6\) (Li and Dewey, 2011) was used to quantify gene abundance. Differential expression analysis was performed using the DESeq2 tool (Love et al., 2014) with FDR<0.05, such that DEGs with \(\log_{2}\text{FC} \geq 1\) and FDR < 0.05 were considered to have a significantly different expression. In addition, KOBAS\(^7\) (Xie et al., 2011) and Goatools\(^8\) was used for functional enrichment analyses, including gene ontology (GO) and Kyoto encyclopedia for genes and genomes (KEGG) to identify significantly overrepresented GO terms and metabolic pathways according to the Bonferroni-corrected \(p\)-value \(\leq 0.05\) compared with the whole-transcriptome background. WGCNA was performed as described by Langfelder and Horvath (Langfelder and Horvath, 2008) and data of all DEGs among the different treatments analyzed. The gene co-expression network was visualized using Cytoscape software (version 3.5.1).

RNA-seq data validation using quantitative real-time PCR

Total RNA was used to synthesize the first strand cDNA using PrimeScript\(^3\) RT reagent Kit with gDNA Eraser Perfect Real Time (Takara, Japan). RT-qPCR was conducted with SYBR Green reagents (Takara, Japan) using an ABI StepOne Plus real-time PCR machine (Applied Biosystems). The PCR program was as follows: 94°C for 5 min, 40 cycles of 30 s at 94°C, 30 s at 60°C and a final melting curve at 65–95°C. GADPH was used as internal reference gene to quantify cDNA. The threshold cycle (\(\Delta\Delta\text{Ct}\)) values of the PCR were averaged and the relative transcript levels were quantified using the \(2^{-\Delta\Delta\text{Ct}}\) method. All primers used for the RT-qPCR are listed in Supplementary Table 1.

Statistical analysis

One-way ANOVA (SPSS 25.0, SPSS Inc., Chicago, IL, United States) and Bonferroni tests \((p < 0.05)\) was used to detect significant differences. All data are presented as means \(\pm\) SD.

Results and discussion

Experimental design and RNA sequence analysis

Six time points and three biological replicates per condition were used to construct RNA-seq libraries for analysis of the effects of NH\(_4^+\) status on gene expression in tea shoots. Quality check were performed to remove reads containing adapter, ploy-N and with low quality from the raw data, leaving an average number of clean reads per library above 6.13 Gb and a total of 130.59 Gb of clean reads. Approximately 91.04–92.25% of the clean reads were successfully mapped to the Camellia sinensis genome and 84.50–85.01% of the clean reads matched unique genomic locations. Each of the transcriptomes had 44.40–45.41% GC content. The values of Q30 were between 93.52 and 94.42% (Supplementary Table 2). These results suggested that the RNA sequencing data used in the present study were highly reliable for de novo assembly and expression analysis. The BLAST tool was used to assess sequence similarity against five public databases: GO, KEGG, NR, Swiss Prot, and Pfam to assign potential functions to the assembled unigenes (Figure 1B).

The results of PCA analysis are shown in Figure 1C. PC1 accounted for 36.98% of the total variance, with clearly separated samples according to the duration of nitrogen deficiency. PC2 accounted for 11.15% of the total variance with samples separated according to different NH\(_4^+\) concentrations, such that the samples with a high concentration showed higher PC2 values (Figure 1C). Three biological replicates of each sample were clustered together, indicating high data reproducibility. Overall, the PCA analysis results showed that both the time and concentration of nitrogen supply could affect the gene expression patterns of tea plants. In addition, development status also affected gene expression. Pearson correlation coefficients of the FPKM distribution among biological replicates of all treatments ranged from 0.958 to 0.997 (Figure 1D), indicating a high reproducibility of the sequencing data.

Analysis of responses to short- and long-term NH\(_4^+\) starvation

Tea polyphenols and free amino acids are the two most important components of tea plants and are affected by nitrogen supply. The content of tea polyphenols (TP) increased with
time, being higher after 7 days. TP content was higher after long-term nitrogen deficiency (L_UN) than after short-term nitrogen deficiency (S_UN) (Figure 2A). Development status had great influence on the content of tea polyphenols and free amino acids. DEGs involved in plant growth were eliminated to enhance the accuracy of experimental results. There were 397 DEGs in short-term nitrogen starvation plants compared with CK1, and most of them (273 genes) were downregulated (Figure 2B). After long-term nitrogen starvation, 172 DEGs were identified, including 92 upregulated and 80 downregulated. Thus, tea plants have a more obvious response to short-term NH$_4^+$ deficiency. The number of DEGs after long-term NH$_4^+$ deficiency may have been fewer because gene expression tended to stabilize after a long period of NH$_4^+$ deprivation.

A total of 14 upregulated and 36 downregulated genes were common to short- and long-term nitrogen deficiency groups (Figure 3A), indicating that duration of nitrogen deficiency affected the expression of different genes. According to GO enrichment analysis, upregulated genes were involved in photosynthesis and protein-chromophore linkage in both long-term and short-term nitrogen deficiency (Figure 3B), suggesting a link between nitrogen deficiency and photosynthesis.
and photosynthesis. Photosynthesis genes, including those encoding photosystem P700 chlorophyll a apoprotein A2 (psaB) (TEA002548), acetyl-CoA carboxylase transferase (chloroplast) (TEA027086) and ribulose bisphosphate carboxylases (Rubisco) (TEA020293) were significantly upregulated by N deficiency (Supplementary Table 3). When nitrogen is scarce, photosynthesis becomes limited and the expression of genes related to the phenylpropanoid pathway (PAL, CHS, and CHI) are known to be up-regulated (Dong et al., 2019). Downregulated genes were enriched in the GO term, cell wall macromolecule catabolic (Figure 3B). Cell wall remodeling represents an important characteristic of plant growth and differentiation (Krouk et al., 2010). The results showed that nitrogen deficiency resulted in slow cell wall remodeling. Biological process in GO terms related to photosynthesis, plant hormone and amino acid metabolism was greatly affected during the early stage of the nitrogen deficiency response. The enhancement of these metabolic pathways may result from the lack of nitrogen disrupting the carbon/nitrogen balance. By contrast, regulations of genes involved in ion transport, lipid and terpenoid metabolism were affected by a long period of nitrogen deficiency. Long-term nitrogen deficiency not only affected carbon fixation in photosynthesis (Makino, 2011), but also inhibited the TCA cycle and fatty acid synthesis (Bouché and Fromm, 2004). Previous studies on Arabidopsis thaliana have shown that PII proteins that regulate nitrogen metabolism was also involved in regulating fatty acid synthesis and metabolism through interaction with the biotin carboxyl carrier protein BCCP (Baud et al., 2010). These results indicate the importance of nitrogen in the growth and metabolism of tea plants.

Analysis of normal and high NH$_4^+$ concentration recovery

Following nitrogen deficiency, different gene expression patterns with different NH$_4^+$ resupply concentrations were found. A total of 3,692 DEGs were found, including 1,268 upregulated and 2,424 downregulated at normal resupply concentration of NH$_4^+$ (Figure 4). With high NH$_4^+$ resupply...
concentration, there were 3,063 DEGs, with nearly equal
to numbers of upregulated and downregulated genes (Figure 4). 
NH$_4^+$ resupply enhanced the changes in gene expression in tea 
plants, reflecting the rapid and obvious response of tea plants to 
NH$_4^+$.

The influence of different NH$_4^+$ concentrations on 
the gene expression patterns of tea plants may be seen 
in the Venn diagram in Figure 5A. At normal NH$_4^+$ 
concentration, there were 1,428 unique genes (320 upregulated 
and 1,175 downregulated) while there were 799 unique 
genes (590 upregulated and 276 downregulated) with high 
NH$_4^+$ concentration resupply. Thus, normal and high NH$_4^+$ 
concentrations may have different gene expression models 
for tea plants. Regardless of NH$_4^+$ concentration, starch 
and sucrose metabolism, phenylpropanoid biosynthesis and 
plant-pathogen interaction were the main response pathways 
after NH$_4^+$ resupply (Figure 5B), suggesting an impact on 
metabolism of nitrogen resupply.

Interestingly, the results showed that the downregulated 
genes with normal NH$_4^+$ resupply were similar to those
upregulated genes with high NH$_4^+$ resupply (Figure 5B). In addition, DEGs were related to plant-pathogen interaction, MAPK signaling, and plant hormone signaling which may account for considerable environmental adaptability. It had also been previously found that the ammonium-specific response is related to biological stress and plant defense (Patterson et al., 2010). High NH$_4^+$ content can protect the plants from pathogens, increase the cross-tolerance to other forms of abiotic stresses and improve the quality of crops (Marino and Moran, 2019). The genes involved in the plant-pathogen interaction, including leucine-rich repeat protein, LRR receptor-like serine/threonine-protein kinase, cyclic nucleotide-gated ion channel (CNGC), disease resistance protein, ethylene-responsive transcription factor, and transcription factor WRKY were significantly highly expressed with NH$_4^+$ resupply. These results have rarely been reported in tea plants. In summary, our results revealed that the signaling role of the NH$_4^+$ molecule.

Identification of gene co-expression modules using weighted gene co-expression network analysis

WGCNA was used to comprehensively understand the gene expression patterns related to NH$_4^+$ treatments in tea plants. After removing the genes with low variability, five modules were identified, as shown in Figure 6. After generating a summary profile for each module, the turquoise module, containing 601 genes, was highly correlated with tea polyphenols, while the brown module, containing 232 genes, was correlated with free amino acids (Figure 6). Tea polyphenols and free amino acids are the two most important components of tea plants. Fewer genes were associated with free amino acids than with tea polyphenols. These results suggested that the effect of NH$_4^+$ on tea polyphenols was more significant compared with that of free amino acids, consistent with previous research (Liu et al., 2017). The top 30 associated genes according to the weighting values were selected from the turquoise and brown modules to construct a network (Supplementary Table 4). Hub genes are genes with the most connections in the network, as shown by their degree.

In the turquoise module, 20 genes were enriched in amino acid metabolism. Both carbohydrate metabolism and biosynthesis of other secondary metabolites contained 17 genes according to KEGG analysis, as shown in Figure 7A. Photosynthesis represents the basis of plant metabolism and energy source and influences tea polyphenols formation. Carbonic anhydrase (CA) (TEA013076) is related to carbon
utilization and was one of the hub genes in photosynthesis. Phytochrome B (TEA031363) was an important gene. Tea polyphenols assist the resistance of plants to damage by external factors, such as UV-B, low temperature, and drought (Zoratti et al., 2014). Genes encoding for the REF/SRPP-like protein (TEA001749), E3 ubiquitin ligase (TEA022683) and carboxylesterase (CXE) (TEA001625) were involved in plant stress resistance (Tong et al., 2017; Cao et al., 2019; Choi et al., 2021). Glutathione S-transferase (GST) (TEA006546) was also a hub gene and involved in normal metabolism of plant secondary products, such as flavonoids and cinnamic acid (Marrs, 1996). Other hub genes identified included glutathione synthetase (GS) (TEA017688) and altered xyloglucan (AXY) (TEA016707) (Figure 7B). These results suggest that nitrogen deficiency may be an abiotic stress that produces reactive oxygen species (ROS). The accumulation of tea polyphenols with high antioxidant activity may contribute to ROS scavenging (Kovacik et al., 2014). Although these genes are not directly involved in the formation of tea polyphenols, they are related to tea plant growth and metabolism, via processes such as photosynthesis, plant stress and carbon and nitrogen balance (Margaria et al., 2014; Wang et al., 2021).

In the brown module, enriched genes were involved in carbohydrate and lipid metabolism and biosynthesis of other secondary metabolites (Figure 8A). The co-expression network is shown in Figure 8B. Cytochrome P450 86A22 (TEA020004) was found to be a hub gene and is a key fatty acid acyl coenzyme essential for the synthesis of esters in petunia (Han et al., 2010). Hub genes included 3-ketoacyl-CoA synthase (KCS) (TEA005835), a rate-limiting enzyme involved in the elongation of very long fatty acid chains (Yang et al., 2021). In rice, the germin-like protein regulates plant height and disease resistance (Banerjee and Maiti, 2010). GDSL esterases/lipases (TEA032710) have also been identified as hub genes and are mainly involved in the regulation of plant development, synthesis of secondary metabolites and the defense response (Chepyshko et al., 2012). Moreover, pathogenesis related protein and germin-like protein are thought to be involved in the response to abiotic stress in plants (Kapoor et al., 2019). In addition, laccase (LAC) (TEA004738) is a hub gene related to lignin synthesis, disease resistance and pigment synthesis (Li and Steffens, 2002; Ranocha et al., 2002; Pourcel et al., 2005). These results demonstrated the complex network system connecting synthesis of free amino acids with many pathways in tea plants.

Validation of the differentially expressed genes by RT-qPCR

Twenty genes from different expression profiles were chosen for RT-qPCR analysis to validate the quality of the RNA-Seq data. Genes belonged to different metabolic pathways, including photosynthesis (photosystem II, chlorophyllase 1), polyphenol synthesis [leucoanthocyanidin reductase (LAR), phalcone synthase (CHS), flavone synthase II], amino acid synthesis (terpene synthase), anti-resistant genes (pathogenesis-related protein, disease resistance protein) and some transporters and transcription factors. The expression value of RT-qPCR was positively correlated with log2 fold change of RNA sequencing results ($R^2 = 0.8338$), indicating the reliability of our transcriptional data (Figure 9). The expression levels of sugar transporter (TEA027183), disease resistance protein (TEA023317) and flavone synthase (TEA012257) genes were increased under high concentration of NH$_4^+$+. High NH$_4^+$+ concentrations, therefore, had a stress effect on tea plants. In summary, tea plants prefer NH$_4^+$+ within a defined concentration range (Ruan et al., 2007).

Data availability statement

The data presented in this study are deposited in the NCBI repository, accession number PRJNA844353 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA844353).

Author contributions

YW and J-XO: data curation. YW, Y-MX, and D-MF: formal analysis. X-CW and X-QZ: funding acquisition, writing—review, and editing. X-CW: project administration. YW: resources and writing—original draft. All authors have read and agreed to the published version of the manuscript.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.963269/full#supplementary-material

SUPPLEMENTARY TABLE 4
Top 30 genes in WGCNA modules.
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