Physiological and Transcriptomic Responses of Antioxidant System and Nitrogen Metabolism in Tomato Roots Treated With Nitrogen Starvation and Re-Supply

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Abstract

Nitrogen (N) is one of the essential macronutrients that plays important roles in plant growth and development. To better understand the response of antioxidant system and N metabolism under N starvation and re-supply condition, physiological and transcriptomic analysis were performed in tomato roots. The malondialdehyde (MDA) and reactive oxygen species (ROS) contents increased significantly in tomato seedlings after N starvation for 24 h. The activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and monodehydroascorbate reductase (MDHAR), the ratio of ASA/DHA and GSH/GSSG, the NO_3^- contents, nitrate reductase (NR) activity were decreased after N starvation treatment and increased after N re-supply for 24 h. Compared with the control, 1766 genes were up-regulated and 2244 genes were down-regulated after N starvation in tomato. These differentially expressed genes (DEGs) are mainly enriched in functional items such as cellular process, metabolic process and catalytic activity. The KEGG pathways revealed that the DEGs were mainly involved in phenpropane biosynthesis, amino sugar and nucleotide sugar metabolism, and N metabolism. The expression patterns of tomato *S/SOD*, *S/CAT*, *S/APX*, *S/MDHAR*, thioredoxin (*S/Trxh*), peroxiredoxin (*S/Prx*) and glutaredoxin (*S/Grx*) genes, and nitrate transporter *S/NRT2.4*, *S/NR*, glutamine synthetase (*S/GS2*), nitrite reductase (*S/NiR*) decreased after N starvation and increased after N re-supply, which were validated by qRT-PCR. Our results provide a basis for understanding the response of tomato to N deficiency and re-supply and a theoretical reference for cultivation regulation.

Introduction

Nitrogen (N) is an extremely important and necessary macronutrient for plant growth, which is also the main component of nucleic acids, proteins, enzymes, and chlorophyll. N has an important role in plant physiological metabolism and growth, and its supply will directly affect plant growth and the accumulation of secondary metabolites (Liu et al. 2017). N containing fertilizers are applied to all important agronomic and horticultural crops to fulfill their requirement, however, only 30–40 % of that fertilizers are utilized by the crops, while the rest of N is lost through leaching or volatilization and causes environmental pollution along with loss of resources (Curci et al. 2017; Xin et al. 2019). The unused compounds released into the environment cause environmental pollution, such as soil acidification, soil hardening, and eutrophication of water (Gutiérrez 2012). Thus, improving the nitrogen use efficiency (NUE) is an important object in the world's agricultural modernization and development. A better knowledge of mechanisms regulating nitrate response in plants will help to develop a strategy to enhance NUE, leading to decrease the use of N fertilizers (Yang et al. 2017).

Organic and inorganic N can be used by plants (Nasholm et al. 2009; Gruffman et al. 2014), inorganic N resources such as nitrate (NO_3^-) and ammonium (NH_4^+) are the major N forms in soil, the former being more abundant in aerobic soils, the latter the major N compound in flooded wetland or acidic soils (Xu et al. 2012; Curci et al. 2017). Nitrogen metabolism is the most important physiological activity in plants, which is closely related to the growth and development, yield and quality of plants (Zhi et al. 2010).

Nitrate as a nutrient, is absorbed by roots through low and high affinity nitrate transporters (NRT1 and NRT2), which is reduced to nitrite by nitrate reductase (NR), and to ammonium by nitrite reductase (NiR). Ammonium is then incorporated into amino acids by glutamine synthetase (GS) and glutamate synthase (GOGAT) (Bolton and Thomma 2008; Rajendran et al. 2008; Pagaling et al. 2014; Chen et al. 2018). As a signal, nitrate induces the expression of a number of genes including NRT1, NRT2, NR, NiR (Bolton and Thomma 2008; Rajendran et al. 2008), GS and GOGAT (Li et al. 2012; Rajendran et al. 2008; Pagaling et al. 2014).

The response of nitrogen metabolism in plants to the change of nitrogen level is multifaceted, and the current research on nitrogen metabolism in plants is mostly concentrated in corn, cereals, wheat and other crops. For example, the activity of nitrate reductase in corn increased with the increase of nitrogen level, which indicated that proper nitrogen application could significantly improve the nitrogen use efficiency (Zhan and Lynch 2015). Studies have shown that the lack of nitrogen in the soil of cereal crops will lead to the change of root length and reduce the protein content of grains (York et al. 2015). In addition, extensive transcriptomic studies have investigated nitrogen metabolisms in plants at various levels, such as time points after N treatments (Krouk et al. 2010), N sources (Patterson et al. 2010), and rates (Wang et al. 2007), in cell (Picault et al. 2002), and tissue types (Wang et al. 2003). In pear roots resupplied by nitrate starvation, the KEGG pathways revealed that 15 unigenes were related to nitrogen metabolism and significantly differentially expressed in response to nitrate starvation and a nitrate re-supply treatment (Chen et al. 2018).

Under the stress of nitrogen deficiency, the dynamic balance between the production and removal of reactive oxygen species (ROS) in plants is broken, which leads to excessive production of ROS in plant cells. If it is not removed in time, it will cause peroxidation of the plasma membrane, thus disrupting the normal metabolism of cells and destroying the integrity of the membrane (Del Río 2015). The types of ROS include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$), alkoxy radical ($RO\cdot$), peroxy radical ($ROO\cdot$) and singlet oxygen (1O_2) (Parisa 2012). In order to ensure their normal metabolic function, plants will form an antioxidant system which can adapt and resist ROS. Antioxidant system is divided into enzymatic antioxidant system and non-enzymatic antioxidant system. Antioxidant enzyme system mainly includes superoxide dismutase (SOD), peroxidase (POD), activity catalase (CAT) ascorbate peroxidase (APX) and other enzymes, non-enzymatic antioxidant systems include AsA, GSH, carotenoids and so on. There are many reports about the antioxidant system of plants under abiotic stress. For example, accumulation of ROS under drought is a prototypic case of stress-induced responses. There are related reports in corn (Avramova et al. 2015), rice (Cai et al. 2015) and *Ailanthus altissima* (Laxa et al. 2019), mulberry (*Morus alba* L.) seedling leaves under NaCl and $NaHCO_3$ stress. Under NaCl stress, the activity of SOD, POD and CAT in leaves increased by some extent. Under $NaHCO_3$ stress, the activity of SOD and POD significantly increased, while that of CAT decreased compared to that of control. The ascorbic acid-glutathione (AsA-GSH) cycle in mulberry seedling leaves was enhancement in both NaCl and $NaHCO_3$ stress (Huihui et al. 2020). Research on rice found that nitrogen stress induces changes in plant ROS content and antioxidant enzyme activity, helping plants resist the damage caused

by oxidative stress (Kumagai et al. 2009). However, there is no report on the effect of nitrogen deficiency and nitrogen re-supply on the antioxidant system of tomato roots.

RNA-Seq, one of next-generation high-throughput sequencing technologies, has been widely used recently, due to low background noise, high sensitivity and reproducibility, great dynamic range of expression and base pair resolution for transcription profiling (Marioni et al. 2008; Quan et al. 2016). Using this technique, transcriptomic profiles of many plants have been dissected under any given conditions, including biotic and abiotic stresses (Nawaz et al. 2018), such as heat stress (Harada and Burton 2020) and drought stress (Hubner et al. 2015). Whole transcriptome analyses using RNA-Seq to examine genes involved in N deficiency have been done for *Arabidopsis* (Shin et al. 2018), maize (Humbert et al. 2013), sorghum (Li et al. 2012; Gelli et al. 2017), cucumber (Xin et al. 2017; Zhao et al. 2015), rice (Yang et al. 2015a; Yang et al. 2015b), wheat (Curci et al. 2017; Wang et al. 2019), and watermelon (Nawaz et al. 2018).

Tomato is cultivated on a commercial scale across the world. According to National Bureau of Statistics of China, annual use of nitrogenous fertilizer exceeds 24 million tons (Xin et al. 2017), a fair share of this nitrogenous fertilizer is utilized for tomato production. However, little research is available on tomato seedlings in response to N deficiency and N re-supply. In this study, an RNA-sequencing approach was applied to gain a comprehensive picture of transcriptional mechanisms underlying the response to N deprivation and N re-supply of tomato roots. In addition, to better characterize the overall effects of these treatments on plant physiological status, the antioxidant enzyme system and N metabolism were also assessed in the experiment. Our results provide a basis for understanding the molecular mechanisms of tomato's response to N recovery after N deficiency stress and laid a theoretical foundation for cultivation.

Material And Methods

Plant Material and Growth Conditions

Tomato seeds were soaked in warm water at 55°C for 1 ~ 2 h, then placed in a petri dish covered with two layers of wet filter paper and put in a thermostat at 28°C for germination. After 2 days, seeds with similar bud potential are sown in perlite with nutrient solution. When tomato seedling grows to a true leaf, they are moved to 4 L square basins for Hoagland's nutrient solution with twelve seedlings each basin (Siddiqi et al. 2002). At three-leaves stage, seedlings were exposed to nitrogen-free nutrients solution (N starvation) for 2 days, after which the tomato seedlings were transferred into Hoagland's nutrient solution with 2.5 mM $\text{Ca}(\text{NO}_3)_2$ and 5mM KNO_3 which contained 10 mM NO_3^- as normal nitrogen. There are 4 treatments in this experiment: (1) normal treatment (CK), tomato nutrient solution cultivation; (2) N deficiency for 2 days, reduction of N elements on the basis of tomato nutrient solution (CK-N); (3) After 2 days of N deficiency, use normal tomato nutrition culture for 6 h (T1); (4) After 2 days of N deficiency, use normal tomato nutrition culture for 24 h (T2). Three replicates were set for each treatment, with 36 tomato seedlings in each treatment. Samples were taken after the treatment, and the young roots of tomato were quickly placed in liquid N for freezing, and then stored in a refrigerator at -80°C.

The lipid peroxidation and ROS accumulation analysis

The lipid peroxidation level was investigated with the malondialdehyde (MDA) contents determined by Draper and Hadley (1990). The ROS accumulation was assayed used the 2, 7-dichlorofluorescein diacetate ($\text{H}_2\text{DCF-DA}$) fluorescence probe. The root tips of the treated seedlings were washed with purified water, and then put into EP tube containing $20 \mu\text{mol}\cdot\text{L}^{-1}$ $\text{H}_2\text{DCF-DA}$ dye for 30 min, washed with $20 \text{ mmol}\cdot\text{L}^{-1}$ HEPES-KOH (PH:7.8) buffer solution for 3 times, each time for 15 min, and the washed root tip samples were photographed under microscope.

Antioxidant enzyme activities analysis

0.2 g of tomato seedling roots were grinded in ice bath in precooled mortar. 1 mL of enzyme extract ($50 \text{ mmol}\cdot\text{L}^{-1}$, PH:7.8, phosphate buffer, $1 \text{ mmol}\cdot\text{L}^{-1}$ EDTA, $1 \text{ mmol}\cdot\text{L}^{-1}$ ASA and 1 % PVP) was add after grinding into 2 mL centrifuge tube, then centrifuged at 4°C and 12000 rpm for 20 min. The supernatant was used to measure enzyme activity, and the experiment was set up in 3 repetitions. The activity of SOD was determined by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) spectrophotometrically at 560 nm (Madhava Rao and Sresty 2000). CAT activity was determined as described by Madhava Rao and Sresty (2000). The activities of APX were determined according to the methods of Yoshiyuki et al. (1981). The activity of monodehydroascorbate reductase (MDHAR) was determined according to Krivosheeva et al. (1996).

Antioxidant substances contents analysis

0.2 g tomato seedling roots were added with 1 mL precooled 5 % sulfosalicylic acid, grounded in ice bath, centrifuged at 12000 rpm for 20 min at 4°C , and the supernatant was taken for later use. The contents of reduced ascorbic acid (AsA) and oxidized dehydroascorbic acid (DHA) were determined by Bipyridine method (Jiang et al. 2001). Oxidized glutathione (GSSG) and reduced glutathione (GSH) were determined by Nagalakshmi and Prasad' DTNB method (Nagalakshmi and Prasad 2001).

Determination of nitrate contents and nitrate reductase activities

The nitrate ion concentration was determined according to Cataldo's methods (CATALDO and Analysis 1975). The activity of nitrate reductase was measured by Living body method (Hageman and Reed 1971). Tomato roots after different nitrogen treatments were rinsed with distilled water and dried with absorbent paper, cut into small pieces with scissors, weighed 0.5 g with a small balance, and put into a triangular flask. First add 1 mL of 30 % trichloroacetic acid solution to the control triangular flask, then add 4 mL of 0.1 mol L^{-1} phosphate buffer (pH:7.5) and 5 mL 0.2 mol L^{-1} KNO_3 to each triangular flask after mixing, put it in a desiccator immediately and evacuate it for 30 minutes. During this period, let in air several times, and then evacuate to make the blade sink completely into the bottom of the bottle, and then put it at 25°C for 30 minutes, add 1 mL of 30 % trichloroacetic acid solution to the triangular flask to stop the reaction. After shaking each triangular flask and let stand for two minutes, then, 2 mL of the

supernatant was treated with 4 mL 1 % sulfonamide and 0.2 % α -naphthylamine before incubating for 15 min in a 35°C water bath with agitation. The nitrate content and nitrate reductase activity were then measured at 410 nm and 540 nm, respectively, using a spectrophotometer.

RNA extraction and detection

RNA was extracted using TransZolUp (TRANS, Company) with approximately 0.1 g sample according to the TRNAS kit instructions. After the RNA extraction is completed, RNA is subjected to 1.0 % gel electrophoresis to ensure the purity and integrity.

Transcriptome sequencing sample preparation and library establishment

After extracting total RNA from the sample, enrich the mRNA with magnetic beads with Oligo (dT), adding fragmentation buffer to the obtained mRNA to make fragments therefore into short fragments, then the mRNA after the fragment is taken as a template. The first strand of cDNA was synthesized with random hexamers. Add buffer, dNTPs, RNase H and DNA polymerase I to synthesize cDNA second chain. The cDNA was purified by QiaQuick PCR test kit and eluted with EB buffer solution, and subjected to end repair. Add base A, add sequencing linker, and then recover the target size fragment by agarose gel electrophoresis, and carry out PCR amplification to complete the whole library preparation work. The constructed library was sequenced by Illumina HiSeq TM. Guangzhou Gidiao Technology company Ltd. was commissioned to complete the library construction, Illumina HiSeq TM sequencing and gene function annotation.

Transcriptome data assembly

First, we filter the Raw data, remove low quality and connectors to obtain Clean data, and use TopHat to compare and remove rRNA-containing reads respectively. Then, the reads of the filtered rRNA are compared to a reference genome. Finally, transcript reconstruction is carried out by cufflinks to obtain all transcripts.

Differential expression gene (DEG) analysis and gene function annotation

The original data is standardized by using the DESeq's own standardized method. In the process of difference analysis, the negative binomial distribution method is used to estimate the distribution of Read count, after evaluating and calculating P value, multiple hypothesis tests are performed on P value to reduce false positives. Differentiated genes were screened according to edgeR's general filtering criteria ($\log_2 |\text{Fold Change}| > 1$ & $\text{FDR} < 0.05$), and the screened differentially expressed genes were enriched with gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) to determine the major metabolic pathways.

Quantitative real-time PCR (qRT-PCR) analysis

Total RNA was extracted and cDNA was synthesized by Prime Script™ RT reagent kit with gDNA Eraser (Perfect Real Time), and fluorescence quantitative assay was carried out by abm® Eva Green qPCR Master Mix-no dye kit. The primers were designed with Software Premier 5.0 based on cDNA Fragments (Table S1). The Pre-denaturation of reaction program was 95°C, 5 min. The number of reaction cycles is 40, the cycle process is 95°C, 10 s, 60°C, 30 s; 72°C, 20 s. The specificity was evaluated by dissolution curve and size estimation of amplification products. The expression level of DEGs was calculated with $2^{-\Delta\Delta C_t}$. Actin gene is used as the standardization of the determination gene. Value of each stage is taken as the average of three technical repetitions for each biological repetition.

Statistical analysis

Three replicates of each sample were used for statistical analysis. Data were analyzed with Student's t-test indicated as follows: *, $P < 0.1$; **, $P < 0.05$. The Graphpad Prism 7.0 software (GraphPad Software, La Jolla California USA, www.graphpad.com) was used for making figures.

Results

Effects of N deficiency and re-supply treatments on lipid peroxidation and ROS accumulation in tomato roots

Membrane lipid peroxidation often occurs when plant organs are aged or damaged under adversity. MDA is the final decomposition product of membrane lipid peroxidation, and its content can reflect the degree of damage to plants under adversity. In this experiment, the MDA content of samples was measured at four treatment time points: CK, CK-N, T1 and T2. After two days of N deficiency treatment, the MDA content increased by 89.90 % compared with that of normal tomato seedlings (Fig. 1a). Compared with the control group, MDA content still increased by 38.57 % after re-supplying N for 6 h, and basically returned to normal level after re-supplying N for 24 h.

The ROS content in tomato roots was labeled with fluorescent probe H₂DCF-DA (Fig. 1b). The fluorescence degree of ROS in roots treated with N deficiency was increased dramatically, which indicated that the oxidative damage caused by N deficiency was aggravated. The fluorescence intensity decreased after re-supplying N, which indicated that the oxidative damage was relieved to some extent after re-supplying N.

Effects of N deficiency and re-supply treatments on antioxidant enzyme activities and ratio of AsA/DHA and GSH/GSSG in tomato roots

As shown in Fig. 2, the activities of SOD, CAT, APX, and MDHAR in tomato roots were all decreased after N deficiency stress compared with the control. SOD activity decreased by 38.02 %, 22.01 % and 19.71 % after 2 days of N deficiency, N re-supply for 6 h and 24 h (Fig. 2a). CAT activity decreased significantly by 48.50 % after N deficiency stress, and continued to decrease to 59.72 % after 6 h of N re-supply (Fig. 2b). APX is an antioxidant enzyme that mainly catalyzes AsA to capture H₂O₂ in chloroplasts and cytoplasm

of plant cells. The APX activity in the roots of tomato seedlings decreased by 32.78 % after N deficiency, and continued to decrease to 43.45 % after 6 h of N re-supply and then increased by 25.02 % after 24 h of N re-supply (Fig. 2c). MDHAR is an important enzyme to regenerate AsA in AsA-GSH cycle. MDHAR activities decreased by 60.13 % after N deficiency and then increased after N re-supply (Fig. 2d).

The ratio of ASA/DHA and GSH/GSSG in tomato seedling roots decreased after N deficiency and then increased after N re-supply (Fig. 2e, f). Compared with the control group, the ratio of GSH/GSSG and ASA/DHA decreased by 29.15 % and 36.22 %, respectively, after 2 days of N deficiency. The ratio of ASA/DHA increased by 16.15 %, 21.59 % and the ratio of GSH/GSSG increased by 8.93 %, and 17.18 %, respectively, after 6 h and 24 h of N re-supply.

Effects of N deficiency and N re-supply treatments on NO_3^- contents, nitrate reductase activity in tomato roots

After N deficiency for 2 days, the NO_3^- content decreased by 39.61 %, compared with the control (Fig. 3a). The NO_3^- content in tomato roots increased by 17.56 % after re-supplying N for 6 h. The N re-supply for 24 h treatments basically restored NO_3^- accumulation to normal levels.

Nitrate reductase (NR) is a key enzyme in the plant nitrate-assimilation process. Therefore, effects of N deficiency and the N re-supplying treatment on the NR activity in tomato roots were investigated. As shown in Fig. 3b, N deficiency treatments significantly reduced the NR activity by 54.78 % in tomato roots. However, the inhibitory effect under conditions of N deficiency was relieved by the N re-supply treatment.

Quality analysis of sequencing results

To investigate the molecular response of N starvation and re-supply to tomato seedling, RNA-seq technology was used. The sample of tomato roots of the control and the treatment group were sequenced by Illumina HiSeq™. In order to ensure the quality of the data, the original data should be quality-controlled before information analysis. Removed reads with adapter, reads with N ratio greater than 10 %, and low-quality reads, CK, CK-N, T1, and T2 have 25515430, 22044082, 21551480, and 21513858 clean reads, and the percentage of Q20 (the quality of the bases after filtering is not less than 20 Proportion) reached 98.32 %, 98.35 %, 98.27 %, and 98.32 %, all of which are greater than 90%. The quality is qualified and meets the requirements of subsequent analysis. In this study, tomato samples of treatments CK, CK-N, T1, and T2 treatments had GC contents of 43.46 %, 43.16 %, 42.96 %, and 42.96 %. Removed reads on the ribosome, four processed Unmapped Reads were got, and the total reads are 25233998, 20919716, 21269156, and 21301956, respectively. Number of reads on the reference genome for unique alignment and the proportion of the total (Unique Mapped Reads) alignment are 22831684 (90.48 %), 19167292 (91.62 %), 19363835 (91.04 %) and 19472134 (91.41 %). The above data will be used for assembly and analysis of the transcriptome (Table 1).

PCA results showed that there was a significant difference in gene expression between the CK group and the CK-N group. The gene expression of the T1 group showed a recovery phenomenon, and the gene

expression of the CK group samples was similar to that of the T2 group samples (Fig. S1). The results showed that the gene expression level basically recovered to that of the CK group of the hydroponic tomato roots resumed N culture for 24 h after 2 days of N deficiency.

Analysis of differences between samples

The R-based software package edgeR was used to process the RNA-seq data for pairwise samples or between groups with significant differences. FDR and $\log_2FC$ were used to screen for differential genes, and the screening conditions were $FDR < 0.05$ and $|\log_2FC| > 1$. As shown in Fig. 4a, compared the CK with CK-N, there were 4010 significantly different genes, of which 1766 were significantly up-regulated and 2244 were significantly down-regulated. Comparing the CK-N and T1 groups, there were 5441 significantly different genes, of which 3534 were significantly up-regulated and 1907 were significantly down-regulated. Comparing the CK-N and T2 groups, there were 4235 significantly different genes, of which 2233 were significantly up-regulated and 2002 were significantly down-regulated. Compared with the T1 and T2 groups, there were 2817 significantly different genes, of which 839 were significantly up-regulated and 1978 were significantly down-regulated.

To identify common and unique DEGs in response to N starvation and re-supply treatments, venn graphs were plotted. We analyzed DEGs that were transcriptionally regulated at different treatment, and a total of 430 common DEGs were identified among the four libraries (Fig. 4b).

GO function annotation analysis of different expressed genes

The number of genes with significant differences in GO function enrichment between each 2 groups is shown in Table S2. These differentially expressed genes are annotated into three directions: biological processes, cell components and molecular functions, involving a total of 45 functional entries. The GO classification charts of Ck-N-vs-T1, Ck-N-vs-T2 and T1-vs-T2 are shown in Fig S2, S3, S4. The GO classification map of CK and CK-N are shown in Fig. 5. In biological processes, differentially expressed genes are most abundant in cellular processes, metabolic processes and single-organism processes. In cell components, differentially expressed genes are mainly concentrated in cell, cell part and membrane. In terms of molecular function, differentially expressed genes are mainly enriched in catalytic activity and binding function items.

Differential gene KEGG Pathway analysis

In order to further understand the biological function of genes and determine the most important biochemical metabolic pathways and signal transduction pathways involved in DEGs, we performed pathway significant enrichment analysis. 7135 differentially expressed genes were annotated in the KEGG database. In CK and CK-N, CK-N and T1, CK-N and T2, T1 and T2, they were annotated to 124, 126, 125, and 113 pathways, respectively. The top 20 pathways with abundant genes are shown in Fig. 6 and Fig. S5, S6, S7. Among them, the differentially expressed genes are mainly involved in phenylpropanoid

biosynthesis, amino sugar and nucleotide sugar metabolism, cysteine and methionine metabolism, starch and sucrose metabolism, N metabolism and other pathways.

Differential expression of N metabolism genes in tomato

The metabolic process of N in plants includes complex mechanisms such as absorption and transport, assimilation and reuse. As shown in Fig. 7, differentially expressed genes were found in this N metabolism pathway. Compared with the control group, 7 DEGs expressions were down-regulated and 1 DEGs expression was up-regulated in hydroponic tomato roots after N deficiency. The down-regulated genes were nitrate transporter (Nrt, Solyc06g010250.3), nitrate reductase (NR, Solyc11g013810.2), nitrite reductase (NiR, 1.7.7.1, Solyc01g108630.3), alpha carbonic anhydrase (14.2.1.1, Solyc09g009830.3). Besides, Beta carbonic anhydrase 5 (14.2.1.1, Solyc09g010970.3), Glutamate dehydrogenase (GDH, 1.4.1.3, Solyc05g052100.3), glutamine synthetase (GS, 6.3.1.2, Solyc01g080280.3). Glutamate synthase 1 (GOGAT, 11.4.1.13 and 1.4.1.14 have the same Nr annotation of Solyc03g083440.3) have up-regulation expression.

The genes that are down-regulated or up-regulated due to N deficiency stress gradually returned to normal expression levels after 1 day of N restoration.

The expression of antioxidant enzyme genes in qRT-PCR and RNA-seq analysis

qRT-PCR analysis was performed on the transcription level of *SISOD*, *SICAT*, *SIAPX* and *SIMDHAR* (Fig. 8). After 2 days of N deficiency in tomato seedlings, the relative expression of *SISOD*, *SICAT*, *SIAPX* and *SIMDHAR* genes in tomato roots decreased by 97.37 %, 94.86 %, 73.83 % and 83.45 %, respectively, compared with the control group. The relative expression of *SISOD* and *SICAT* genes increased by 6.55 and 3.76 times respectively after N re-supply for 6 h. The relative expression of *SIAPX* and *SIMDHAR* genes decreased by 74.63 % and 51.24 % respectively after N re-supply for 6 h. The relative expression of *SISOD*, *SICAT*, and *SIMDHAR* genes increased by 7.54, 8.53 and 1.91 times, and the relative expression of *SIAPX* genes decreased by 43.89 %, after N re-supply for 24 h.

The mRNA expression of *SITrxh*, *SIPrx* and *SIGrx* was also analyzed by qRT-PCR in the roots of tomato seedling after the N deficiency and re-supply. *SITrxh*, *SIPrx* and *SIGrx* expression decreased by 56.73 %, 93.27 % and 87.31 %, respectively, compared with the control group after 2 days of N deficiency. *SITrxh* expression decreased by 53.74 % compared with the control group after 6 h of N re-supply. *SIPrx* and *SIGrx* expression increased by 4.77 and 1.03 times of the control group after 6 h of N re-supply. The mRNA expression of *SIPrx* and *SIGrx* increased significantly after 24 h of N re-supply.

We analyzed the transcriptome, and the results are basically consistent with the real-time PCR data (Table S3), indicating that the transcriptome data is reliable.

The expression of N metabolism genes in qRT-PCR and RNA-seq analysis

Compared with the control group, the transcription levels of *SINRT2.4*, *SINR*, *SINiR* and *SIGS* in hydroponic tomato seedlings were down-regulated by 65.43%, 13.22%, 73.11% and 54.72%, respectively, after N starvation (Fig. 9). Compared with the control group, the transcription level was up-regulated by 1.17, 2.24, 2.15 and 0.59 times after N re-supplying for 24 h. Compared with the control group, the transcription level of *SIGOGAT* gene after N starvation treatment was increased by 2.19 times, and after 24 h of N re-supplying, the transcription level was only 1.07 times that of the control group, which basically recovered to the level of the control group.

Discussion

N is an important and most abundantly required macronutrient for plant growth and development. N availability directly affects chlorophyll formation and photosynthetic assimilation (Nawaz et al. 2017; Luo et al. 2013; Takei et al. 2001; Tischner 2001; Colla and Science 2010). In our experiment, the ROS contents increased after N starvation, which indicated that the balance of ROS was disrupted by the N starvation. MDA content is the embodiment of the degree of cell membrane peroxidation in plants. In this experiment, the content of MDA in the root system of N-deficient tomato was significantly higher than that of the control. The MDA content was also markedly increased in the leaves and roots of wheat seedlings during N starvation (Guo et al. 2014).

Plants have developed an antioxidant machinery that includes the activity of ROS detoxifying enzymes [e.g., SOD, APX, CAT, and Prx], as well as antioxidant molecules such as ascorbic acid (ASA) and glutathione (GSH) that are present in almost all subcellular compartments (Cassia et al. 2018). Nitrate starvation repress genes related to the cytoskeleton and ROS detoxification (Ravazzolo et al. 2020). In our experiment, compared with the control group, SOD, CAT activities decreased after 2 days of N deficiency (Fig. 2). The APX in tomato gradually lost its activity after N deficiency treatment, which may be due to excessive ROS attacking biological functional molecules in defense system under N deficiency stress (Hasanuzzaman et al. 2019). In the cycle of AsA-GSH in plants, AsA is oxidized to MDHA, and MDHA regenerates AsA under the action of MDHAR. MDHAR activity decreased after N deficiency, N re-supply for 6 h and 24 h, compared with the control group (Fig. 2d). Previous results showed that AsA synthesis in tomato roots was damaged after N deficiency (Hasanuzzaman et al. 2019). In our study, the ratio of AsA/DHA decreased after N starvation. Transcripts involved in antioxidant responses were strongly up-regulated when *T. suecica* was cultured under N starvation (Lauritano et al. 2019). However, in our study, the expression of antioxidant enzymes of *SISOD*, *SICAT*, *SIAPX*, *SIMDHAR* were all decreased after N starvation in tomato roots, indicating the decreased ROS detoxification capability.

Most of the studies have implicated N transport genes, N assimilation genes, and GS/GOGAT cycle genes involved in NUE. The amount of nitrate ion in tomato root decreased after N deficiency treatment, which is consistent with the previous studies that the N starvation condition led to a marked reduction in total N content in roots (Curci et al. 2017). Under N limitations, reduced nitrate and total N concentrations in the N-starved rapeseed plants were also detected (Zhang et al., 2018). In addition, the nitrate ion content basically returned to normal level after supplying N for 24 h. This indicated that tomato roots in N

deficiency state can uptake an adequate supply of nitrate within 24 h of its being N resupplied, which are consistent with earlier reports (Chen et al. 2018; Richard-Molard et al. 2008). In previous reports, NR activity decreased under N deficiency stress in cotton (Iqbal et al. 2020), rice (Sinha et al. 2018), and wheat (Sinha et al. 2015), which was consistent with the results in this paper. In this study, tomato seedlings after two days of N deficiency, GOGAT genes were up-regulated, while the expression of *SlGS*, *SlNR*, and *SlNiR* genes were down-regulated in tomato roots. Previous research showed that N starvation caused a significant decrease both in transcript levels in NR, NiR, GS, and GOGAT activities. Potassium nitrate treatment restored NR, NiR, GS, and GOGAT expressions and activities (Balotf et al. 2016). These results demonstrate that N transport and assimilation are adjusted by the GS/GOGAT cycle in response to N availability. Re-supplying nitrate to the nitrate-starved tomato plants quickly and significantly increases the transcription levels of many genes, such as nitrate transporters, NR, and NiR, in the first 24 h, but this up-regulation trend lasts for 48 h and then subsides (Scheible et al. 2004).

RNA-seq was a suitable transcriptomic technique to study the molecular mechanism on plant growth and development, plant stress resistance and other aspects under any given condition. Recently, this method has been also performed on some crops, viz rice, sorghum, cucumber and watermelon (Yang et al. 2015a; Zhao et al. 2015; Nawaz et al. 2018; Gelli et al. 2014), to investigate the mechanism of N deficiency tolerance. Re-supplying nitrate to the nitrate-starved Arabidopsis seedlings within 30 min, markedly and extensively alters the expression levels of genes involved in primary and secondary metabolism, cellular growth, hormone responses, protein synthesis, signal transduction and transcriptional regulation (Scheible et al. 2004). Iqbal et al. (2020) reported the genotypic difference in transcriptome profile using two cotton genotypes differing in NUE under N starvation and resupply treatments. A large genetic variation existed in DEGs related to amino acid, carbon, and nitrogen metabolism. The identification of key genes underlying the response to N starvation may enable novel approaches to increase NUE and to improve plant resilience to nutritional stresses (Ravazzolo et al. 2020). In tomato, 97 genes are differentially expressed when nitrate is resupplied to nitrate-starved tomato plants within 1–96 h (Wang et al. 2001). Functional analysis of the N-starvation response proteins suggested their involvement in protein synthesis and fate, metabolism, and defense of young rice leaves grown under nitrogen-starvation conditions (Kim et al. 2011). N compound metabolism, carbon metabolism, and photosynthesis were mostly enriched in durum wheat under N starvation (Curci et al. 2017). Metabolic process, cellular process, and transport were enriched in rice roots and shoots under N-free conditions (Yang et al. 2015a). In our study, comparing the two groups of tomato seedling roots treated with N deficiency and N re-supply, the DEGs were involved in metabolic process, nutrient reservoir activity and catalytic activity responding to N deficiency and N re-supply treatment.

KEGG pathway analysis can help us to further understand the biological functions of genes and how these genes interact (Kanehisa et al. 2004). In our study, the differentially expressed genes are mainly involved in the pathways of phenylpropanoid biosynthesis, amino sugar and nucleotide sugar metabolism, cysteine and methionine metabolism, starch and sucrose metabolism, N metabolism and other pathways. In previous report, cotton under N starvation and re-supply treatment, enriched to a pathway similar to the results of our experiment (Iqbal et al. 2020). The KEGG pathways revealed that 15

unigenes, including one NRT gene, two NR genes, one NiR gene, two GDH genes, six GS genes and three GOGAT genes, were related to nitrogen metabolism and significantly differentially expressed in response to nitrate starvation and a nitrate re-supply treatment (Chen et al. 2018). From these pathways explained the possible roles of N deficiency and re-supply on the N metabolism of tomato.

In sum, antioxidant enzyme system and N metabolism were analyzed based on environmental conditions that restored N supply after two days of N deficiency in tomato seedlings by physiological and RNA-seq analysis. This study provided a valuable resource for better understanding of tomato seedling roots in responses to N starvation and re-supply and for understanding the genes and pathways involved.

Declarations

Author Contributions

H.N. X designed the project. Y.Y D, M.Q. W, F.Z.W conducted the experiments and analyzed the data. Y.Y D wrote the article. J.P. Y and K.Z. L helped in the writing of the article. All authors agreed on the final content of the article.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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Tables

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

Figures

Fig.1

Figure 1

Effect of N starvation and re-supply on the lipid peroxidation (a) and ROS accumulation. All the results represent mean $\pm$ standard deviation (SD) of three biological replicates. Data were analyzed with Student's t-test compared with control under similar conditions and indicated by *, $P < 0.1$; **, $P < 0.05$.

Fig.2

Figure 2

Effect of N starvation and re-supply on the activities of SOD (a), CAT (b), APX (c), MDHAR (d) and the ratio of AsA/DHA (e) and GSH/GSSG (f) in the roots of tomato seedling. All the results represent mean $\pm$ standard deviation (SD) of three biological replicates. Data were analyzed with Student's t-test compared with control under similar conditions and indicated by *, $P < 0.1$; **, $P < 0.05$.

Fig.3

Figure 3

Effect of N starvation and re-supply on the nitrate content (a) and nitrate reductase activities (b) in the roots of tomato seedling. All the results represent mean $\pm$ standard deviation (SD) of three biological replicates. Data were analyzed with Student's t-test compared with control under similar conditions and indicated by *, $P < 0.1$; **, $P < 0.05$.

Fig.4

Figure 4

Analysis of significant differences between samples and Venn map of DEGs among different combinations of comparisons.

Fig.5

Figure 5

Functional annotation of assembled sequences based on gene ontology (GO) categorization. The unigenes are summarized into three main categories: biological process molecular function and cellular components.

Fig.6

Figure 6

The KEGG metabolism pathway categories of DEGs of CK and CK-N. The pathway names are provided in the vertical axis, rich factor in the horizontal axis, size of the point represents the number of DEGs, and the color of the dot represents the q value.

Fig.7

Figure 7

Differentially expressed genes involved in nitrogen metabolism pathway of CK VS CK-N and CK-N VS T2. Green represents genes down-regulated, and red represents genes up-regulated.

Figure 8

Quantitative real-time PCR validation of SISOD (a), SICAT (b), SIAPX (c), SIMDHAR (d) SITrx (e), SIPrx (f), SIGrx (g) in tomato seedling roots under N starvation and N re-supply treatments. All the results represent mean $\pm$ standard deviation (SD) of three biological replicates. Data were analyzed with Student's t-test compared with control under similar conditions and indicated by *, $P < 0.1$; **, $P < 0.05$.

Fig.9

Figure 9

Quantitative real-time PCR analysis of the nitrogen metabolism expression of SINRT2.4 (a), SINR (b), SIGS (c), SINiR (d) SIGOGAT (e) in tomato roots in response to N starvation and N re-supply. All the results represent mean $\pm$ standard deviation (SD) of three biological replicates. Data were analyzed with Student's t-test compared with control under similar conditions and indicated by *, $P < 0.1$; **, $P < 0.05$.

Supplementary Files

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