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Research article

Effects of nitrate deficiency on nitrate assimilation and chlorophyll synthesis of detached apple leaves

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ABSTRACT

Nitrogen is one of the most important nutrients for plant growth and development. Nitrate nitrogen (NO_3^--N) is the main form of nitrogen taken up by plants. Understanding the effects of exogenous NO_3^--N on nitrogen metabolism at the gene expression and enzyme activity levels during nitrogen assimilation and chlorophyll synthesis is important for increasing nitrogen utilization efficiency. In this study, cell morphology, NO_3^--N uptake rates, the expression of key genes related to nitrogen assimilation and chlorophyll synthesis and enzyme activity in apple leaves under NO_3^--N deficiency were investigated. The results showed that the cell morphology of apple leaves was irreversibly deformed due to NO_3^--N deficiency. NO_3^--N was absorbed slightly one day after NO_3^--N deficiency treatment and effluxed after 3 days. The relative expression of genes encoding nitrogen assimilation enzymes and the activity of such enzymes decreased significantly after 1 day of NO_3^--N deficiency treatment. After treatment for 14 days, gene expression was upregulated, enzyme activity was increased, and NO_3^--N content was increased. NO_3^--N deficiency hindered the transformation of 5-aminobilinic acid (ALA) to porphobilinogen (PBG), suggesting a possible route by which NO_3^--N levels affect chlorophyll synthesis. Collectively, the results indicate that NO_3^--N deficiency affects enzyme activity by altering the expression of key genes in the nitrogen assimilation pathway, thereby suppressing NO_3^--N absorption and assimilation. NO_3^--N deficiency inhibits the synthesis of the chlorophyll precursor PBG, thereby hindering chlorophyll synthesis.

1. Introduction

Nitrogen is an essential macronutrient for plant growth and development and is involved in the synthesis of nucleotides, proteins, enzymes and chlorophyll in plants (Marschner, 1995). NO_3^--N, which is taken up through energy expenditure and transport via transpiration, is the primary form of nitrogen absorbed and utilized by plants (Tang et al., 2013). After NO_3^--N is absorbed, most of it is transferred to aboveground mesophyll cells through the xylem to be reduced. This process involves the absorption, assimilation, transport and utilization of NO_3^--N, among which assimilation is the most critical step and one of the most important limiting factors (Xuan et al., 2017). NO_3^- is reduced to NH_4^+ by nitrate reductase (NR) and nitrite reductase (NiR) in the cytoplasm and then transported to the chloroplast. Glutamic acid and glutamine are then generated via assimilation through glutamine synthetase (GS) and glutamate synthase (GOGAT) (Maeda et al., 2014; Plett et al., 2016). Generally, when the nitrogen supply is insufficient, enzyme activities, such as those of NR, NiR, GS, and GOGAT, are

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Abbreviations: NO_3^--N, nitrate nitrogen; ALA, 5-aminobilinic acid; PBG, porphobilinogen; NR, nitrate reductase; NiR, nitrite reductase; GS, glutamine synthetase; GOGAT, glutamate synthase; Urogen III, uroporphyrinogen III; UROS, uroporphyrinogen III synthetase; ALAD, 5-aminolevulinic acid dehydratase; UE, upper epidermis; PP, palisade parenchyma; SP, spongy parenchyma; LE, lower epidermis; GluTR, Glu-tRNA reductase enzyme

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affected by aging and stress. For example, Balotf et al. (2016) demonstrated that the transcription levels of genes encoding NR, NiR, GS and GOGAT in wheat were significantly reduced after 7 days of NO_3^--N deficiency treatment. Chloroplasts serve as a nitrogen storage unit, with more than 70% of nitrogen in the plant being stored in these organelles (Han et al., 2016). As chlorophyll is a nitrogen-containing compound synthesized in chloroplasts, NO_3^--N deficiency directly affects its synthesis.

An important pigment involved in photosynthesis, chlorophyll converts light energy into chemical energy, providing resources for plant growth development and representing the basic food source on Earth (Cocaliadis et al., 2014; Meng et al., 2018). The synthesis of chlorophyll in Arabidopsis leaves has been well studied: the chlorophyll biosynthetic pathway requires 15 steps, 15 enzymes, and 27 genes encoding enzymes, all of which have been cloned in Arabidopsis (Meskauskiene et al., 2001). The synthesis of ALA is a crucial factor controlling chlorophyll synthesis (Gough et al., 2003), and conversion of ALA to PBG and the synthesis of uroporphyrinogen III (Urogen III) directly affect the chlorophyll content (Bollivar et al., 2006). *HEMA*, a key gene controlling chlorophyll synthesis, encodes glutamyl-tRNA reductase, which catalyzes the synthesis of ALA from L-glutamyl-tRNA. ALA is converted to PBG by 5-aminolevulinic acid dehydratase (ALAD), which is encoded by the *HEMB* gene. PBG is converted to protoporphyrin IX through a 5-step catalytic reaction under the catalysis of *HEMD*-encoded uroporphyrinogen III synthetase (UROS) (Beale, 2005; Alawady et al., 2005). In the last step of chlorophyll biosynthesis, magnesium (Mg)-chelatase inserts Mg^{2+} into protoporphyrin IX.

Nitrogen is one of the most important nutrients for plant growth and senescence, and it is mainly utilized and distributed in the form of glutamine and NO_3^--N (Diaz et al., 2008; Fan et al., 2009). According to Balotf (2015), NR, NiR, GS, and GOGAT activities and the transcriptional levels of the corresponding genes in wheat seedlings are significantly reduced by nitrogen deficiency. In general, the activity of nitrogen metabolism enzymes is significantly related to the synthesis of chlorophyll (Zhao et al., 2008). Under NO_3^--N deficiency, nitrogen is transported to vigorous tissues and storage organs, chlorophyll is degraded, and leaves develop a senescence phenotype (Qiu et al., 2015). Leaves are the main organ of photosynthesis in plants, and NO_3^--N deficiency affects leaf senescence and leads to decreased photosynthetic capacity (Bassi et al., 2018). Thus, increasing nitrogen use efficiency in plants is the foundation of modern agriculture.

Nonetheless, excessive nitrogen application aggravates environmental issues, causing pollution of the environment and water (Adelaide, 2007). To date, studies on the effects of nitrogen deficiency have mainly focused on leaf senescence and root growth (Park et al., 2018; zhang et al., 2017), whereas few have reported its effects on nitrogen assimilation and absorption and chlorophyll synthesis. In the present study, the effects of NO_3^--N deficiency on leaf cell morphology, nitrogen assimilation and chlorophyll synthesis were examined under tissue culture conditions to elucidate the physiological mechanism and molecular basis of NO_3^--N assimilation and chlorophyll synthesis in apple leaves and to enrich the theory of nitrogen absorption in apple leaves.

2. Materials and methods

2.1. Plant materials and growth conditions

The experimental materials used in this study were leaves of tissue-culture plantlets of *Malus x domestica* 'Gala 3′ obtained in May 2018. 'Gala 3′ tissue-culture plantlets uniformly grown for one month were selected at the State Key Laboratory of Crop Sciences and the College of Horticultural Science and Engineering of Shandong Agricultural University (Tai'an Shangdong). The functional leaves were removed, and the tip and petiole of the leaves were scratched along the vertical vein direction using sterilized surgical blades. The leaves were laid flat on MS differentiation medium under suitable nitrogen conditions (NO_3^-concentration: 0.039 mol/L, total N concentration: 0.060 mol/L). Ten leaves were precultured in the dark for 3 days and then transferred to light for 7 days. The leaves were transferred to MS differentiation medium (CK, NO_3^-concentration: 0.039 mol/L, total N concentration: 0.060 mol/L) and MS NO_3^--N deficiency differentiation medium (T, NO_3^-concentration: 0 mol/L, total N concentration: 0.021 mol/L) with NH_4Cl and KCl instead of NH_4NO_3 and KNO_3 and cultured for three weeks. Three biological replicates were included for each treatment. The culture conditions in the solar greenhouse were 14 h of light (24 °C), 10 h of darkness (22 °C), relative humidity of approximately 70%, and illumination intensity of 20,000 lx. After 0, 1, 3, 7, 14 and 21 days, leaves from the different treatments were immediately frozen in liquid nitrogen and stored at −80 °C for further analysis.

2.2. Observation of leaf cell morphology

The apple leaves contacting the medium were cut vertically into small pieces of approximately 1 cm^2 along the vertical direction of the veins. The materials were quickly placed in FAA fixing solution (3.7% methanol, 50% acetic acid, 5% glacial acetic acid, 41.3% water) under vacuum for 24 h (Jin, 2007). The plant material was removed from the fixing solution, smoothed with a surgical blade in the fume hood, and then dehydrated with different gradients of alcohol and waxed as follows: 75% alcohol for 4 h→85% alcohol for 2 h→90% alcohol for 2 h→95% alcohol for 1 h→absolute ethanol I for 30 min→absolute ethanol II for 30 min→alcohol and benzene for 10 min→xylene I for 10 min→xylene II for 10 min→wax I for 1 h→wax II for 1 h→wax III for 1 h. The wax-impregnated plant material was then embedded in an embedding machine (Leica RM2235, Germany) (Ye and Feng, 2006). After placing melted wax into the embedding frame, the tissue was inserted into the embedding frame, and the corresponding label was attached before the wax solidified (Li, 2009). After cooling on a −20 °C freezing platform, the wax block was removed from the embedding frame and trimmed after it solidified. Each wax block was sliced into 3 μm-thick slices using a semiautomatic wheel slicer. The slices were floated in the 40 °C water of the spreader, the tissues were flattened on the slide, and the slices were baked in an oven at 60 °C. After the water had evaporated and the wax had melted, the slices were stored at room temperature for subsequent dyeing. Before dyeing, the slices were treated with an alcohol gradient as follows: xylene I for 20 min→xylene II for 20 min→absolute ethanol I for 5 min→absolute ethanol II for 5 min→75% alcohol for 5 min. The slices were then washed with sterile water. Safranine staining was carried out according to Huang and Du's method (1991): 1% saffron was used to stain for 1–2 h, and then the samples were washed with sterile water to remove excess dye. The slices were decolorized in 50%, 70% and 80% gradient alcohol for 5 s. For green staining, a 0.5% dye solution was used for 30–60 s, and then the samples were dehydrated with absolute ethanol. The slices were placed into clean xylene for 5 min and then sealed with neutral resin. The microstructure of the leaves was observed and photographed under a optical microscope (Nikon YS100, China).

2.3. Determination of the NO_3^- content and flow rate in leaves

The NO_3^--N content was determined according to Ren (2005). The NO_3^-dynamic flow rate in leaves was measured using a nondamaging micromeasurement system (NMT 100 Series, Younger USA LLC, Amherst, MA 01002, USA) at the Agricultural College of Shandong Agricultural University. Leaves contacting the medium were cut into pieces of approximately 0.5 cm^2, placed at the bottom of a dish and fixed with resin. The leaves were then immersed in a test solution containing 0.1 mM KNO_3 and 0.1 mM $CaCl_2$ (pH 5.8) for 30 min. The test solution was replenished, and the sensor tip was placed at approximately 50 μm from the surface of the leaves to begin testing. Each leaf was measured for 5 min to obtain NO_3^- flow data (Han et al., 2016; Zheng et al.,

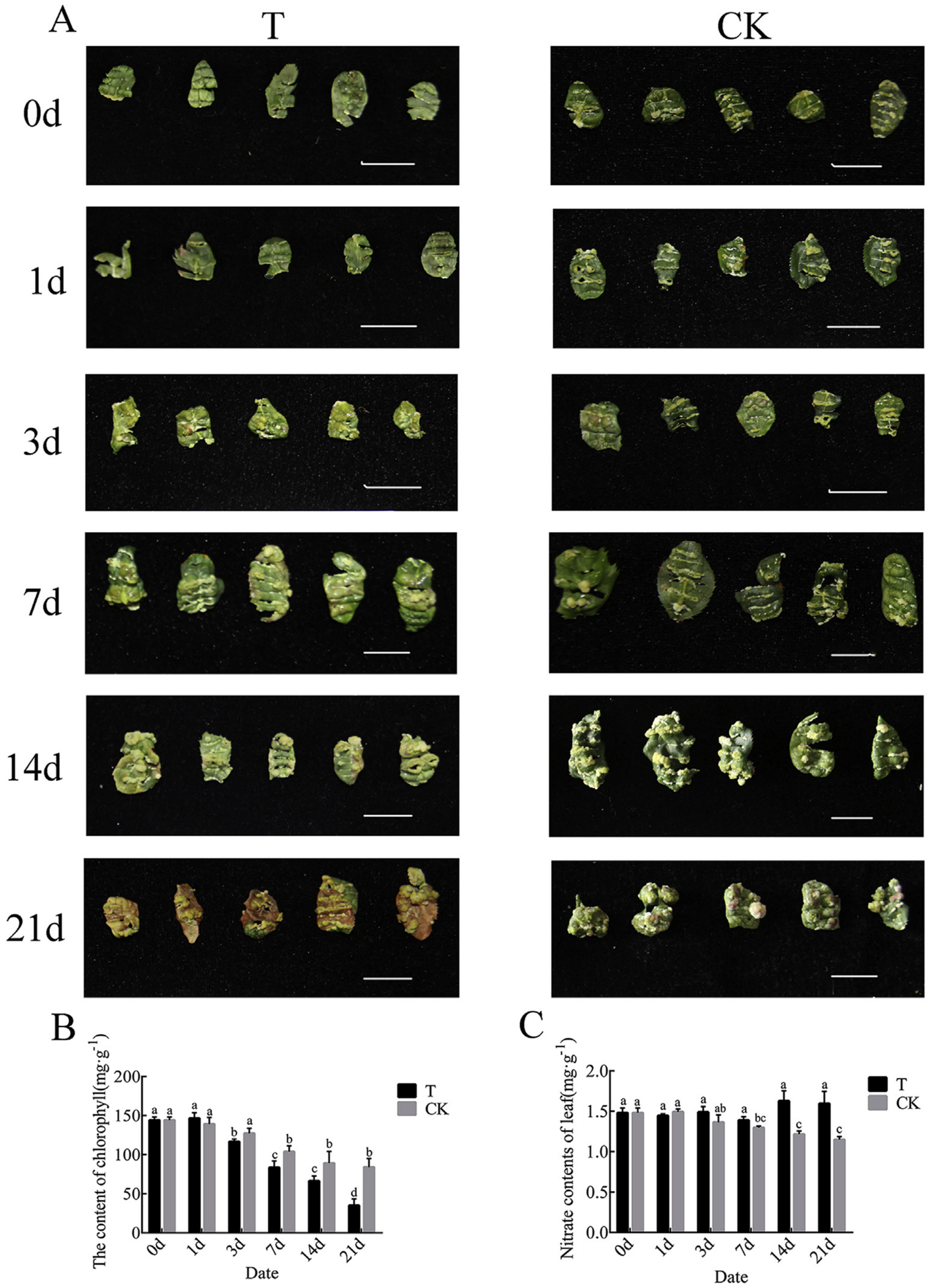

Fig. 1. Effects of NO_3^--N deficiency on leaf growth, chlorophyll content and NO_3^--N content in detached apple leaves. The phenotype of apple leaves (A). Changes in chlorophyll content (B) and NO_3^--N content (C). Different letters above the bars indicate significant differences between means at $P < 0.05$. T: NO_3^--N deficiency treatment; CK: Control. These same abbreviations are used below.

2013). Six biological replicates were analyzed for each group (each representing a different period). Positive values of NO_3^- flow rate represented efflux, and negative values represented absorption.

2.4. Determination of NO_3^--N assimilation enzyme activity

The activity of NO_3^--N assimilation enzymes was determined using a commercial kit. One-gram samples of apple leaves (in three biological replicates) contacting the medium were ground adequately with liquid nitrogen. Nine milliliters of 1 x PBS buffer (pH 7.2–7.4) was used to completely rinse out the mortar contents. The material was centrifuged for 20 min (3000 r/min), and the supernatant was carefully collected. NR, NiR, GS and GOGAT kits were purchased from Tongwei Shanghai (Nos. ml48521j, ml69312j, ml15789j, and ml25784j, respectively; Shanghai Tongwei). The absorbance (OD value) of each well at 450 nm was measured using an iMARKTM microplate reader (Serial No. 14833, Japan). Analyses were carried out within 15 min after adding the termination solution.

2.5. Determinations of chlorophyll content, key enzyme activities and substances in the chlorophyll synthesis pathway

The chlorophyll content was determined according to the method of Zhao (2002). The determination of the activities of key enzymes and the contents of intermediates in the chlorophyll synthesis pathway was consistent with the method for determining NO_3^--N assimilation enzyme activity. The contents of ALA, PBG, and Urogen III and the activities of the key enzymes ALAD and UROS were measured using commercial kits (Nos. ml25421j, ml12365j, ml23458j, ml25781j, and ml98541j, Shanghai Tong Wei).

2.6. RNA extraction

Total RNA was extracted from apple leaves on days 0, 1, 3, 7, 14 and 21 after treatment using the RNAprep Pure TIANGEN Kit (TIANGEN, DP441, Beijing). The quality and quantity of the RNA were determined using a Kaiao K5500 spectrophotometer (Kaiao, Beijing) followed by 1% agarose gel electrophoresis (18S and 28S bands). The integrity and concentration of the RNA samples were detected using the Agilent 2100 RNA Nano 6000 Assay Kit (Agilent Technologies, CA, USA) to ensure high-quality RNA samples.

2.7. Quantitative real-time PCR (qRT-PCR)

To reveal the molecular basis of the effects of nitrate nitrogen on leaf metabolism, we selected genes encoding key enzymes in the NO_3^--N assimilation and chlorophyll synthesis pathways. Total RNA was reverse transcribed (1 μg per sample) in a 20-μl reaction using a cDNA reverse transcription synthesis kit (RR047A, TaKaRa, China). The specific primers used for the investigated genes are listed in Table S1. The SYBR® Premix Ex Taq™ (Tli RNaseH Plus) Kit (Thermo Fisher) was employed for quantitative fluorescence PCR. The reaction system included the following: 12.5 μL of SYBR® Premix Ex Taq (2 ×), 1 μL each of forward and reverse primers, 1 μL of cDNA, and ddH_2O to yield a final volume of 25 μL. The experimental design included 3 technical replicates. The quantitative fluorescence PCR conditions were as follows: 35–40 cycles of predenaturation at 95 °C for 30 s, denaturation at 95 °C for 5 s, and annealing at 60 °C for 30 s. After the reaction, a fluorescence curve and melting curve were obtained, and the comparative Ct ($2^{-\Delta\Delta Ct}$) method was utilized for data analysis (Livak et al., 2001).

3. Results

3.1. Effects of NO_3^--N deficiency on leaf growth

As shown in Fig. 1A, the color of the apple leaves changed significantly with increasing duration of NO_3^--N deficiency, changing from dark green to light green and, finally, to yellow green. After a slight increase, the chlorophyll content gradually decreased, becoming lower than that of the control group after 3 days of treatment (Fig. 1B). At the end of treatment, the callus at the leaf wound was yellow and did not exhibit normal growth. Over the treatment period, the NO_3^--N content in the leaves of the treatment group first decreased and then increased, although the variation was not significant. The NO_3^--N content of the control leaves gradually decreased over time, and after 3 days, it was significantly lower than that of the treatment group (Fig. 1C).

3.2. Effects of NO_3^--N deficiency on leaf microstructure

Leaves are the main organs through which plants cope with environmental changes, and the anatomical structure of leaves directly reflects their growth status (Luna et al., 2017). NO_3^--N deficiency had a significant effect on the anatomical structure of apple leaves (Fig. 2). The normal leaf anatomical structure is composed of obvious palisade tissue and spongy parenchyma; the mesophyll close to the upper epidermis consists of three layers of palisade tissue comprising compactly arranged cylindrical and thin-walled small cells. After three days of NO_3^--N deficiency treatment, the volume of spongy parenchyma had decreased, and the intercellular space had increased to form air chambers. After 7 days of treatment, the palisade cells displayed no significant stratification and were irregular arranged. The number of spongy parenchyma cells had decreased, and more air chambers had formed. At this time, the leaves contacting the culture medium were light green, and their growth was inhibited (Fig. 1A). After 14 days of treatment, the palisade cells were malformed, the cell gap had expanded further, and the number of spongy parenchyma cells had further reduced.

3.3. Effect of NO_3^--N on NO_3^- flow in apple leaves

The change in NO_3^- flux in apple leaves at different times under NO_3^--N deficiency was measured using a nondamaging micro-measurement system (Fig. 3A). The results are shown in Fig. 3B. After one day of NO_3^--N deficiency, apple leaves absorbed NO_3^--N at a rate 13.87 pmol/(cm^2·s). However, after three days of treatment, the apple leaves showed an NO_3^- efflux effect, and there was a significant tendency toward efflux with treatment for 14 days; relative to the control rate, the absorption rate was −80.52 pmol/(cm^2·s) at this time. When treated for 21 days, the absorption rate of the treatment group reached the maximum value of the control group, which was 152.90 pmol/(cm^2·s). In general, the apple leaves exhibited short-term NO_3^- absorption after NO_3^--N deficiency treatment. Efflux in the treatment group reached its maximum at 14 days.

3.4. Effects of NO_3^--N deficiency on nitrogen assimilation enzyme activities in apple leaves

After treatment with NO_3^--N deficiency, the activities of nitrogen assimilation enzymes first decreased and then increased (Fig. 4). NR and NiR are mainly involved in the reduction of NO_3^--N in mesophyll cells. After one day of NO_3^--N deficiency, NR activity had decreased significantly, but it did not differ significantly from the control activity at this time point. After 7 days of treatment, NR activity had increased but was significantly lower than that of the control (Fig. 4A). NiR catalyzes nitrite reduction. After one day of NO_3^--N deficiency, NiR activity had decreased significantly, but it had increased gradually after three days and reached the pretreatment level at the end of treatment; as a result the time at which NR activity reached the pretreatment level was delayed (Fig. 4B). GS and GOGAT are mainly involved in primary nitrogen assimilation in chloroplasts. After one day of NO_3^--N deficiency treatment, GS activity had decreased; it then showed a gradual increase after three days. The activity of GS peaked at 14 days after treatment and was significantly higher at this time than before treatment (Fig. 4C). With NO_3^--N deficiency, GOGAT activity first decreased significantly; it had increased slightly by 14 days of treatment, though it was lower than that of the control at this time (Fig. 4D). Overall, nitrogen assimilation enzyme activity was significantly decreased after 1 day of NO_3^--N deficiency treatment; although it increased slightly over 14 days of treatment, it remained lower than that of the control.

3.5. Effects of NO_3^--N deficiency on the synthesis of ALA and the transformation of ALA to PBG in apple leaves

ALA, PBG and Urogen III are important precursors in chlorophyll synthesis. As shown in Fig. 5A, ALA first increased and then decreased with increasing duration of NO_3^--N deficiency, and the peak value was reached after 14 days of treatment. In contrast, there was no significant change in PBG content over the course of treatment (Fig. 5C). The activity of ALAD had increased slightly on the 7th day of treatment, but the level did not differ among the remaining time points. (Fig. 5B). In general, NO_3^--N deficiency did not significantly increase ALAD activity, which was significantly lower under NO_3^--N deficiency than under control treatment, directly leading to accumulation of ALA. In

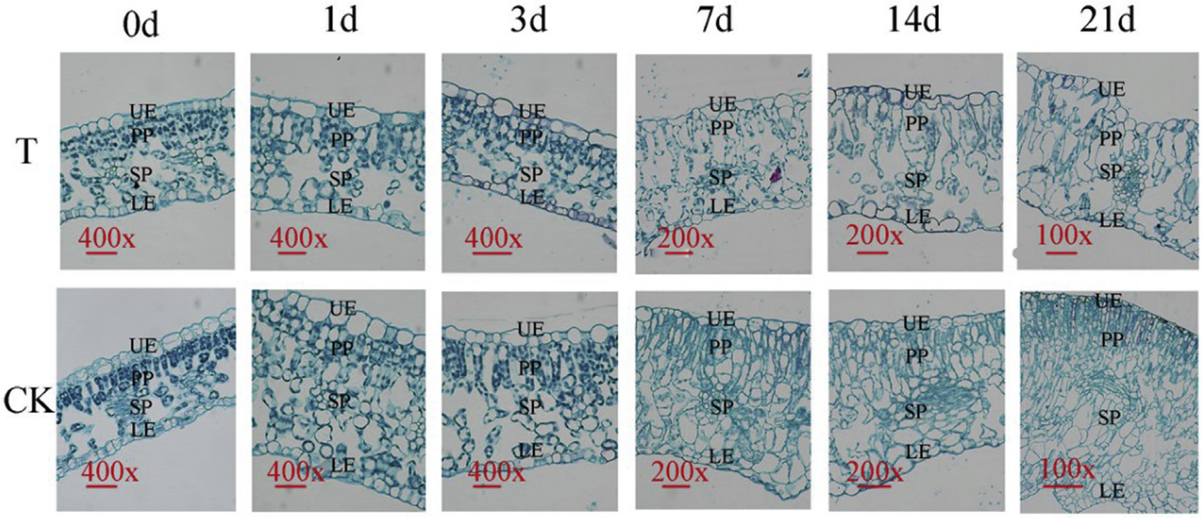

Fig. 2. Micrographs of cross sections of apple leaves under NO_3^--N deficiency and normal conditions. UE: upper epidermis; PP: palisade parenchyma; SP: spongy parenchyma; LE: lower epidermis.

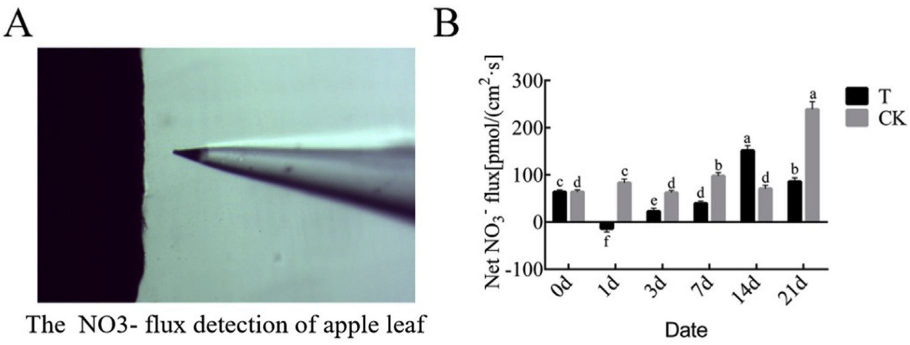

Fig. 3. NO_3^- uptake flux in apple leaves. NO_3^- flux detection of apple leaves (A). Flow of NO_3^- in apple leaves (B). A positive value indicates absorption, and a negative value indicates efflux.

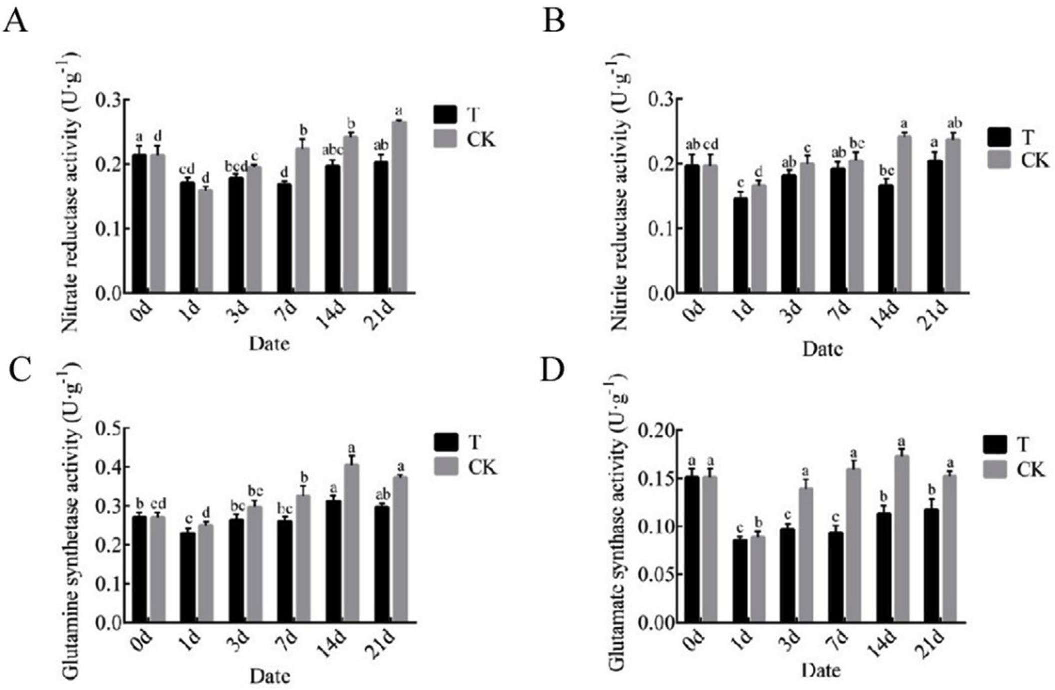

Fig. 4. Enzyme activities in the nitrogen assimilation pathway. Activity of NR (A). Activity of NiR (B). Activity of GS (C). Activity of GOGAT (D).

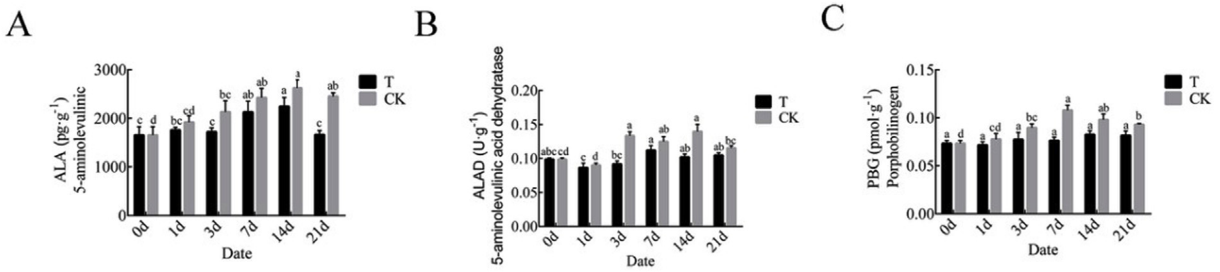

Fig. 5. Effects of NO_3^--N on the synthesis of ALA and the conversion of ALA to PBG. Content of ALA (A). Activity of ALAD (B). Content of PBG (C).

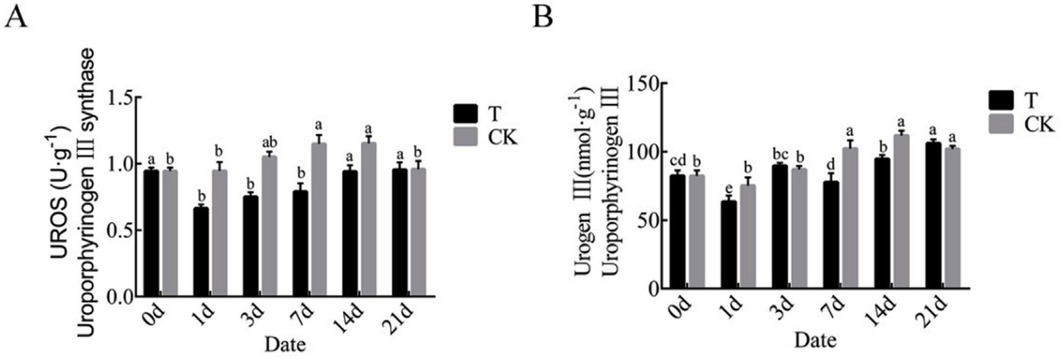

Fig. 6. Effect of NO_3^--N on Urogen III synthesis. Activity of UROS (A). Content of Urogen III (B).

addition, the transformation of ALA to PBG was inhibited.

3.6. *Effects of NO_3^--N deficiency on the synthesis of Urogen III and UROS activity in apple leaves*

Urogen III is formed in the UROS-mediated coupling reaction of ALAD and PBG (Aiko et al., 2013). As indicated in Fig. 6, the changes in Urogen III and UROS were consistent with each other under NO_3^--N deficiency treatment, first decreasing and then increasing. However, after 14 days of NO_3^--N deficiency, UROS activity did not differ significantly from that before treatment (Fig. 6A). Correspondingly, the content of Urogen III reached its maximum at 21 days (Fig. 6B). The Urogen III content and UROS activity were lower than the control levels at 1–14 days of NO_3^--N deficiency. Overall, UROS activity decreased significantly and was lower than that of the control after NO_3^--N deficiency treatment. With the prolongation of treatment time, the activity of UROS increased, and it reached the pretreatment level at 14 days, which accelerated the formation of cyclic Urogen III catalyzed by hydroxymethyl cholane. The content of Urogen III also increased significantly, but there was no significant difference between the treatment and control at 21 days.

3.7. *Effects of NO_3^--N deficiency on nitrogen isozyme and chlorophyll synthase gene expression*

Fig. 7 shows that the expression levels of nitrogen assimilation enzyme genes (*MdNR, MdNIR, MdGS, and MdGOGAT*) first decreased and then increased under NO_3^--N deficiency treatment and were lower than those of the control. After one day of NO_3^--N deficiency treatment, expression of the nitrogen isozyme gene had decreased significantly; it then increased over time, peaking at 14 days of treatment. At this time, the relative expression was 0.48-, 1.16-, 1.32- and 1.16-fold that of the pretreatment samples. Moreover, the expression levels of key enzymes in the chlorophyll synthesis pathway were lower than the corresponding control levels after NO_3^--N deficiency treatment. The relative expression of *MdHEMA1* and *MdHEMA2* first increased and then decreased, peaking at 7 days and 1 day after treatment, respectively. In addition, the relative expression of *MdHEMB1* and *MdHEMB2* was upregulated after 3 days of treatment and significantly lower than that of the pretreatment samples after 7 days. The relative expression of *MdHEMD* was similar to that of the nitrogen assimilation enzyme genes. The relative expression levels of the genes encoding nitrogen assimilation enzymes and key genes in the chlorophyll synthesis pathway were lower than those in the control group, and the peak expression of key enzyme genes in the chlorophyll synthesis pathway occurred before that of the nitrogen assimilation enzyme genes. In summary, among the time points, one day of NO_3^--N deficiency treatment had the greatest effects on the expression of these genes.

4. Discussion

4.1. *Effects of NO_3^--N deficiency on the growth and microstructure of apple leaves*

Leaf development is accompanied by physiological and biochemical changes, and abiotic factors play a major role in this process (Woo et al., 2004; Meng et al., 2016). Nitrogen is one of the most important nutrients in plant growth and development, and its lack can affect leaf growth (Bouchet et al., 2014). In the present study, the contact area of the leaf with the culture medium changed from dark green to light green after 3 days of NO_3^--N deficiency. After 21 days of treatment, the leaves were withered and yellow, and senescence and death phenotypes appeared. These findings indicated that the NO_3^--N supply is closely related to the normal growth of apple leaves. However, this study did not find that leaf senescence was accompanied by an increase in leaf epidermal thickness (Sorin et al., 2015); this result may have been due to the inhibition of leaf growth and development under nitrate deficiency. Although leaf thickness did not increase, the internal cell morphology changed significantly. Indeed, with the prolongation of NO_3^--N deficiency, the three layers of palisade tissue became one layer. Moreover, the small parenchymal cells that compose the palisade tissue became larger, and the number of spongy tissue cells decreased, resulting in the formation of larger air chambers. These results indicate

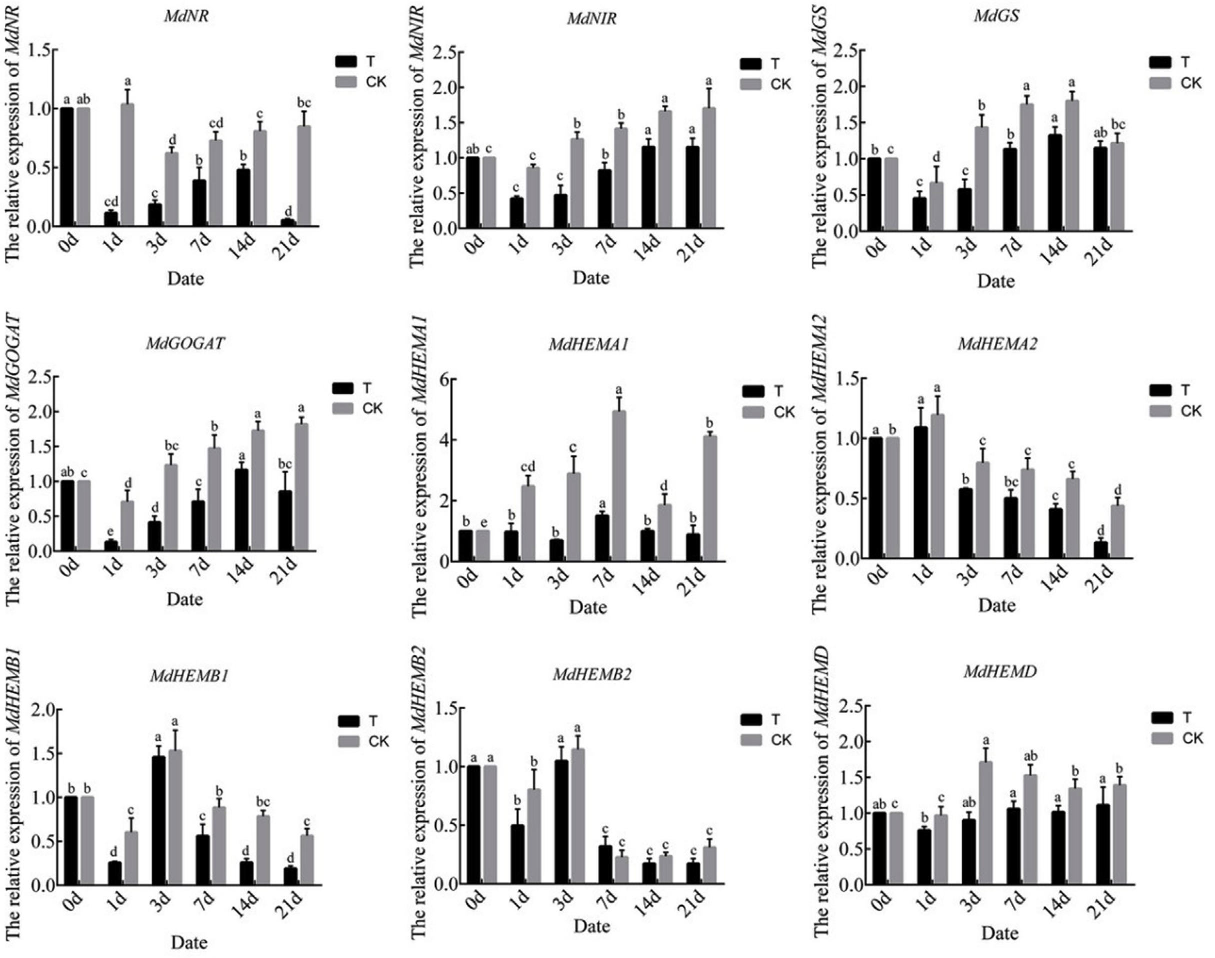

Fig. 7. Quantitative RT-PCR results of genes encoding enzymes involved in nitrogen assimilation and key enzymes of the chlorophyll synthesis pathway. *NR*: Nitrate reductase; *NiR*: Nitrite reductase; *GS*: Glutamine synthase; *GOGAT*: Glutamate synthase; *HEMA*: Glutamyl-tRNA reductase; *HEMB*: 5-Aminolevulinate dehydratase; *HEMD*: Uroporphyrinogen III synthase.

that NO_3^--N deficiency had significant effects on leaf structure, suggesting that NO_3^--N plays important roles in leaf growth and development.

4.2. Effects of NO_3^--N deficiency on NO_3^--N absorption and assimilation

Exogenous nitrogen is one of the main factors affecting nitrogen uptake, and the flow rate of NO_3^- can dynamically reflect nitrogen absorption by plants (Hawkins and Robbins, 2010). Previous studies in barley have shown that NO_3^--N uptake is negatively regulated: when NO_3^--N was continuously supplied to seedlings, the uptake rate increased, but with an increase in NO_3^- concentration, uptake decreased (Siddiqi et al., 1989). In the present study, apple leaves displayed short-term absorption of NO_3^- after one day of NO_3^--N deficiency treatment. Because the test solution contained 1 mM NO_3^-, the NO_3^- that was absorbed in the short term after one day of NO_3^--N deficiency treatment was likely derived from the test solution. However, an insufficient nitrogen supply limits the absorption of NO_3^--N. After 3 days, the efflux rate increased gradually, and the rate peaked at 80.52 pmol/(cm^2·s) after 14 days. At this time, the content of NO_3^--N in the leaves was increased, whereas that in the callus was decreased, which may be related to the leaf nitrogen status. After 14 days of NO_3^--N deficiency treatment, the NO_3^--N in the callus was transferred to the leaves, resulting in an efflux peak. The molecular mechanisms responsible for these findings require exploration.

NR is induced by NO_3^-, and its activity is mainly affected by the concentration of NO_3^- (Kovács B et al., 2015). NiR and NR participate in the process of reducing NO_3^- to NH_4^+ in coupled regulation. In our study, NR activity was positively correlated with NO_3^--N content in apple leaves after one day of NO_3^--N deficiency, but it was lower than that of the control at this time point. This finding is consistent with previous studies reporting that nitrogen isozyme activity and mRNA expression levels are related to external NO_3^--N concentrations (Balotf et al., 2016). NR and NiR activities were significantly reduced from pretreatment levels on the first day after NO_3^--N deficiency treatment, with decreases of 19.72% and 25.89%, respectively. The relative expression levels of *MdNR* and *MdNIR*, which encode NR and NiR, were also significantly decreased, by 88.54% and 57.88%, respectively, after one day of NO_3^--N deficiency treatment, which may have been due to the reduced nitrogen availability under NO_3^--N deficiency (Takatoshi et al., 2016). After treatment with NO_3^--N deficiency, the relative expression level of the nitrogen isozyme gene and the activity of the isozyme were decreased. When inorganic nitrogen is converted to other forms of nitrogen, the availability of NO_3^--N decreases. However, the nitrogen in the callus tissue was continuously transferred to the leaves, which led to an increase in the NO_3^--N content and NR and NiR activities. In higher plants, GS and GOGAT assimilate ammonia into amino acids for plant absorption and utilization in leaves (Ángel et al., 2018). As the concentration of NH_4^+ is closely related to GS and GOGAT enzyme activities (Pinto et al., 2014), external NO_3^- affects GS and GOGAT activities by influencing the reduction of NH_4^+. In the present study, the relative expression of *MdGS* and *MdGOGAT* and in the activities of GS and GOGAT first decreased and then increased with increasing treatment time. The relative expression of *MdGS* and

Table 1
Chlorophyll biosynthetic pathway.

Step	Enzyme name	Biosynthetic product
1		L-glutamyl-tRNA
2	Glutamyl-tRNA reductase	L-glutamic acid-1-semialdehyde
3	Glutamate 1-semialdehyde aminomutase	5-aminolevulinic acid
4	5-Aminolevulinate dehydratase	Porphobilinogen
5	Hydroxymethylbilane synthase	Hydroxymethylbilane
6	Uroporphyrinogen III synthase	Uroporphyrinogen III
7	Uroporphyrinogen III decarboxylase	Coproporphyrinogen III
8	Coproporphyrinogen oxidative decarboxylase	Protoporphyrinogen IX
9	Protoporphyrinogen oxidase	Protoporphyrin IX
10	Magnesium chelatase D subunit Magnesium chelatase H subunit Magnesium chelatase I subunit	Mg-protoporphyrin IX Mg-protoporphyrin IX Monomethyl ester
11	Mg-protoporphyrin IX methyltransferase	Divinyl protochlorophyllide
12	Mg-protoporphyrin IX monomethylester cyclase	Protochlorophyllide
13	NADPH: protochlorophyllide oxidoreductase	Chlorophyllide a
14	Chlorophyll synthase	Chlorophylla
15	Chlorophyllide a oxygenase	Chlorophyllide b

MdGOGAT and the activities of GS and GOGAT had decreased after 1 day of NO_3^--N deficiency treatment but then peaked after 14 days. The results of this study reveal the relationships between the concentration of exogenous NO_3^--N and both nitrogen assimilation gene expression and nitrogen assimilation activity. According to our results, the gene expression and enzyme activity of nitrogen isozyme were significantly decreased after NO_3^--N deficiency treatment. At 14 days of treatment, nitrogen assimilation gene expression was upregulated, and enzyme activity was increased, although it was lower than the control activity.

4.3. Effects of NO_3^--N deficiency on chlorophyll synthesis

The process of chlorophyll synthesis is shown in Table 1 (Xing et al., 2010). Interruption at any one of these steps hinders the synthesis of chlorophyll, and the synthesis pathway of ALA is considered an important step in the synthesis of chlorophyll precursors (Simon et al., 2003;Masuda and Fujita, 2008). To explore the role of NO_3^--N in chlorophyll synthesis, we examined the effect of NO_3^--N deficiency on ALA synthesis. The results showed that the relative expression of *MdHEMA1* and *MdHEMA2*, which encode Glu-tRNA reductase enzyme (GluTR), was inhibited and that the content of ALA was reduced after NO_3^--N deficiency treatment relative to the control levels. The ALA content peaked after 14 days of treatment, representing an increase of 35.77% from the content before treatment. However, the increase in ALA content did not cause increases in the levels of other intermediates in chlorophyll synthesis (Nguyen et al., 2016; Wu et al., 2018). The genes *MdHEMB1* and *MdHEMB2*, which encode ALAD, were upregulated at 3 days after treatment but downregulated at the other periods. This finding is consistent with the results of Chen's study, which revealed that short-term stress can cause the upregulation of chlorophyll synthesis genes (Chen et al., 2014). Overall, the ALAD activity and PBG content did not change significantly following NO_3^--N deficiency treatment. However, the synthesis of Urogen III was directly affected by the PBG content and UROS activity. In this study, the relative expression of *MdHEMD*, which encodes the UROS enzyme, and UROS activity first decreased and then increased over time, though there was no significant difference in their levels between pretreatment and the end of treatment. The content of Urogen III gradually increased over time, which may have been due to the slowing of PBG anabolism after NO_3^--N deficient treatment, and the mechanism of negative feedback regulation promoted the synthesis of Urogen III. Therefore, we speculate that NO_3^--N deficiency affects the conversion of ALA to PBG, thus affecting the formation of precursors for chlorophyll synthesis and chlorophyll synthesis itself.

5. Conclusions

In summary, NO_3^--N plays important roles in the transcription and posttranscriptional regulation of nitrogen assimilation and chlorophyll synthesis pathway enzymes (Fig. 1C).

This study provides insight into the transcriptional and post-transcriptional regulatory pathways of nitrogen assimilation and chlorophyll synthesis due to NO_3^--N deficiency, providing a theoretical basis for improving the utilization efficiency of nitrogen fertilizer.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.07.007.

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Author contributions

BBW, WX and DSG designed the research. BBW and XHZ performed the experiments. BBW and HYS analyzed the data. XDC, DSG and XWC contributed new models. BBW wrote the manuscript, and WX and LL revised the intellectual content of this manuscript. All authors read and approved the final manuscript.

Conflicts of interest

All authors declare that the research was conducted without any conflicts of interest.

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