TRANSCRIPTOMIC ANALYSIS OF ADAPTIVE RESPONSES TO NITROGEN DEFICIENCY IN QUINOA

YANG, L. Y.[1] – YANG, X. L.[1] – ZHOU, X. Y.[1] – YANG, Y. S.[2*]

[1]*College of Life Science, Shanxi Normal University, Taiyuan 030031, P. R. China*

[2]*Shanxi Electronic Science and Technology Institute, 501 West Binhe Road, Linfen, Shanxi 041000, P. R. China*

[*]*Corresponding author*
wyfighting_123@163.com

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Abstract. Quinoa (*Chenopodium quinoa*) is a promising pseudocereal crop that grows well under nutrient-poor conditions. To elucidate the transcriptional changes and the associated alterations in physiological parameters in response to low nitrogen (LN) and nitrogen starvation (NS) conditions, we identified differentially expressed genes (DEGs) in LN and NS seedlings compared with CK seedlings and investigated related photosynthetic parameters changes and biomass accumulation. Compared with CK seedlings, 2404 and 4362 DEGs at day 5, and 10,466 and 11,107 at day 30 were identified, respectively. DECs related to antioxidant biosynthesis were up-regulated after 5 days of LN or NS treatment, and DEGs related to carbon metabolism were up-regulated after 30 days. Five genes related to Psb and ten genes related to Rubisco were highly expressed which contributed to the improved biomass under low-nitrogen conditions. Five out of the seven CA genes involved in nitrogen metabolism were strongly induced. After 30 days, the photosynthetic parameters in LN and NS treatment were decreased compared with those in the CK, and the LN treatment had a smaller effect on photosynthetic parameters than the NS treatment. After 120 days, quinoa in LN treatment group showed higher NUE than the CK, in which the grain yield and nitrogen concentration were 67.4% and 58.2% of those in the CK, respectively, with 20% nitrogen application of the CK. These results provide insight into the molecular and physiological mechanisms associated with the low-nitrogen adaptations of quinoa, which could be useful for breeding crop cultivars that tolerate nitrogen deficiency.
Keywords: *Chenopodium quinoa, nitrogen-poor conditions, transcriptome, differentially expressed genes, photosynthesis*

Introduction

Nitrogen (N), as a vital nutrient, plays a crucial role in the growth and development of plants, making it a key factor in enhancing crop productivity (Li et al., 2024). Plants need sufficient nitrogen to synthesize amino acids, proteins, chlorophyll and other substances for growth and development, so it is important to provide enough nitrogen fertilizer. However, the current reliance on environmentally harmful synthetic fertilizers to achieve global grain yields highlights the necessity for increased nitrogen use efficiency in order to ensure future agricultural sustainability (Liu et al., 2020). Soil fertility is fundamental to crop productivity, and nitrogen (N) is usually the most growth-limiting plant nutrient. The main source of nitrogen for non-leguminous plants is nitrogen fertilizer. However, nitrogen application is expensive and unsustainable. In fact, crops often have a low nitrogen use efficiency (NUE); the global average NUE was estimated to be 0.4% in 2010 (Zhang et al., 2015). High N fertilizer application together with low crop NUE leads to environmental pollution. Therefore, it is critical to develop crops with greater NUE to maximize growth and yield. For this, it is imperative to understand how plants

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appropriately adjust their metabolism to different N levels, especially to low N application.

Quinoa (*Chenopodium quinoa* Willd.) is a resilient and high-yielding pseudo-crop that produces grains with a superior protein content and a well-balanced amino acid composition compared to other cereals. It is a halophytic pseudocereal crop, has been cultivated in the Andes Mountains for approximately 7000 years. It has garnered global interest due to its exceptional nutritional profile and adaptability to diverse environmental conditions, including nutrient-poor, marginal agroecosystems (Hinojosa et al., 2018). This adaptability is particularly noteworthy in the Southern Altiplano region of Bolivia, where the organic matters in the soil is minimal (approximately 0.7%) (Jacobsen et al., 2011). It is demonstrated that quinoa responds positively to nitrogen fertilization, but differences in yield were found among irrigated and rainfed conditions. Quinoa can produce 1850 kg grains ha^{-1} with 50 kg N ha^{-1} under irrigated conditions, and 670 kg grains ha^{-1} with 15 kg N ha^{-1} in rainfed conditions (Cárdenas-Castillo et al., 2021). Li et al (2023) found that differentially expressed genes (DEGs) and differentially expressed metabolites (DEMs) of quinoa are affected by the concentration of nitrogen, and quinoa seedlings were able to cope in different nitrogen fertilizer environments by primarily regulating arginine biosynthesis.

Low-N tolerance in plants is a complex trait, and numerous physiological and phenotypic indices are influenced by nitrogen deficiency, which can be utilized to assess low-N tolerance (Deng et al., 2023). The hypothesis put forth in this study posits that quinoa may possess certain genetic traits and physiological adaptations that allow it to thrive in nitrogen-deficient environments. To investigate this, researchers conducted an analysis of transcriptomic profile changes and associated physiological characteristics in quinoa seedlings subjected to varying nitrogen levels.

Materials and Methods

Plant material and treatments

The experiment was carried out in the College of Life Sciences, Shanxi Normal University, China from 2019 to 2021. A quinoa line ZK7 that grown in Jingle County, Shanxi Province, China (N50°14' E112°23') provided by the Institute of Crop Science, Shanxi Agricultural University, China was adopted. They were selected for uniform size and sterilized for 20 min with 10% NaClO, rinsed thoroughly with distilled water, and incubated in petri dishes covered with wet filter paper at 25°C until germination. The germinated seeds were then moved to pots (20-cm diameter) filled with sand and watered with Hoagland solution every two days in a growth chamber under a 16-h-light/8-h-dark photoperiod at 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ intensity, 18–22°C, and 75% humidity, until the seedlings had four to six leaves. The uniform seedlings were then subjected to three nutrient solution treatments with different nitrogen concentrations: complete nutrient solution (CK) with a nitrogen content of 15 $mmol \cdot L^{-1}$ [0.5 M $Ca(NO_3)_2$ and 0.5 M KNO_3], low-nitrogen solution (LN) with a nitrogen content of 3 $mmol \cdot L^{-1}$ [0.1 M $Ca(NO_3)_2$ and 0.1 M KNO_3], and nitrogen starvation solution (NS) with 0.5 M KCl and 0.5 M $CaCl_2$ in place of $Ca(NO_3)_2$ and KNO_3 to balance the ionic strength. Hoagland solution formula was used to prepare the nutrient solution. In addition to nitrate, the nutrient solution also contained 0.5 M KCl, 0.5 M $CaCl_2$, 2.5 mM $MgSO_4 \cdot 7H_2O$, 2.3 mM KH_2PO_4, 0.02 mM Fe-EDTA, 0.05 mM H_3BO_3, 7 μM $MnSO_4$, 0.3 μM $CuSO_4 \cdot 5H_2O$, 0.8 μM $ZnSO_4$, and

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0.5 μM H_2MoO_4. The pH was maintained at 5–6. The culture solutions were refreshed every 2 or 3 days. After 5 and 30 days of treatment, the samples were taken.

Sampling and measurement methods

RNA extraction, library preparation, and sequencing

Quinoa samples were collected after 5 and 30 days of treatment for RNA sequencing. At each sampling time, equal amounts of young leaf tissue were harvested from the 3rd or 4th leaf of each treatment group for RNA extraction. Total RNA was isolated using Trizol, and the integrity, purity, and concentrations of the RNA were evaluated via an Agilent 2100 Bioanalyzer equipped with an RNA 6000 Nano Chip (Agilent Technologies, Palo Alto, CA, USA).

High-quality RNA with 28S:18S > 1.5 and a 260/280 absorbance ratio of 1.8–2.2 was used for library construction and sequencing. For each RNA sample preparation, 1 μg of total RNA was utilized as input material. Sequencing libraries were produced using the NEBNext UltraTM RNA Library Prep Kit for Illumina (NEB, USA), following the manufacturer's instructions. In brief, mRNA was isolated from total RNA using poly-T oligo-attached magnetic beads. First strand cDNA was synthesized using a random hexamer primer and M-MuLV Reverse Transcriptase. This was followed by second strand cDNA synthesis, carried out using DNA Polymerase I and RNase H. The cDNA library was assessed using the Agilent Bioanalyzer 2100 system. Upon successful cluster generation, the library were sequenced on an Illumina Novaseq 6000 platform to generate paired-end reads of 150 bp. Clean data (clean reads) were obtained by discarding those containing adapters, ploy-N and low quality reads. These clean reads were then mapped to the reference genome sequence (ASM168347v1) using Hisat2 tools soft. Gene expression was calculated as fragments per kilobase of transcript per million fragments mapped (FPKM). Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway were analyzed based on RNA-seq data. GO enrichment analysis was conducted using Blast2GO (https://www.blast2go.com/) with FDR < 0.05. Differentially expressed genes (DEGs) were determined using DESeq2 with fold change ≥ 2 and FDR < 0.05 as the screening standard. Fluorescent quantitative reverse transcription PCR (qRT-PCR) were used to verify the results of RNA-Seq (*Supplementary 1*).

Enzyme activity

The activity of ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) and phosphoenolpyruvate arboxylase (PEPC) were determined 30 d after treatment. The top fully expanded leaves were homogenized in cold sodium phosphate buffer (0.1 M, pH 7.0, 4°C) and centrifuged, the supernatant was collected for enzyme activity analysis with MEIMIAN KITS (Jiangsu Kete Biotechnology Co., Ltd, China), and the specific detective methods were performed according to the kit instructions.

Chlorophyll fluorescence parameters and chlorophyll content

Chlorophyll fluorescence parameters were recorded with a PAM-2100 portable chlorophyll fluorometer (Germany) 30 days after treatment. Three plants were randomly selected from each treatment group, the top fully expanded leaf of plants was kept in darkness for 20 min, and the chlorophyll fluorescence parameters were recorded. The chlorophyll content was then measured spectrophotometrically according to Zhang et al. (2009).

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Biomass and nitrogen content

To compare seedling growth profiles under different N treatments, 10 seedlings from each treatment group were randomly collected on the 30th, 120th day of treatment. Dry weight of shoots and roots were recorded, and root-to-shoot ratio was calculated with the following formula: root/shoot ratio (R/S) = root dry weight/shoot dry weight. Total nitrogen content was measured using the Kjeldahl Nitrogen method (Li et al., 2012).

Data analysis

Values are means ± SD of independent replicates for each treatment. Differences among treatments were analyzed by one-way ANOVA, Duncan's multiple range test, SPSS 18.0.

Results

Nitrogen deficiency induces global transcriptome changes in quinoa

Raw RNA-seq data were generated with 39.5–55.4 million reads per library. After quality control, 93.9–94.1% reads per library were retained and 95.54–96.66% were mapped to the reference genome (ASM168347v1), with 86.24–91.06% reads uniquely mapped. The total mapped reads, uniquely mapped reads, and multiple-mapped reads are summarized in *Table 1*.

Table 1. *Summary of RNA and mapping using the quinoa (ASM168347v1) genome as a reference*

Total reads	Clean reads	GC (%)	Total mapped reads (%)	Uniquely mapped reads (%)	Multiple mapped reads (%)
43 666 970	21 833 485	44.13	95.54	87.59	7.95
39 524 672	24 305 980	43.96	95.84	86.39	9.45
48 611 960	19 762 336	43.99	95.92	86.24	9.69
49 542 228	24 771 114	43.83	96.14	91.06	5.08
55 391 736	22 149 363	44.03	96.66	90.33	6.34
44 298 726	27 695 868	44.19	96.36	88.06	8.30

*LN5d and LN30d represent quinoa seedlings under low nitrogen conditions for 5 and 30 days, respectively. NS5d and NS30d represent quinoa seedlings under nitrogen starvation conditions for 5 and 30 days, respectively

We identified 2404 DEGs in the LN treatment group after 5 d, with 1313 up-regulated and 1091 down-regulated DEGs compared with the CK group. In the NS treatment group, 1962 up-regulated and 2400 down-regulated DEGs were identified. More DEGs in the LN and NS treatment groups were identified at 30 d than at 5 d. There were 10,466 and 11,107 DEGs in the LN and NS treatment groups, respectively, with 5185 and 5798 up-regulated and 5283 and 5309 down-regulated DEGs, respectively (*Figure 1A, Table S1*). There were 1222 DEGs shared in the LN and NS treatment groups at 5 d, and 7002 shared at 30 d (*Figure 1B, Table S2*). These results indicate that more DEGs are induced after longer exposure to N deficiency and by greater N deficiency.

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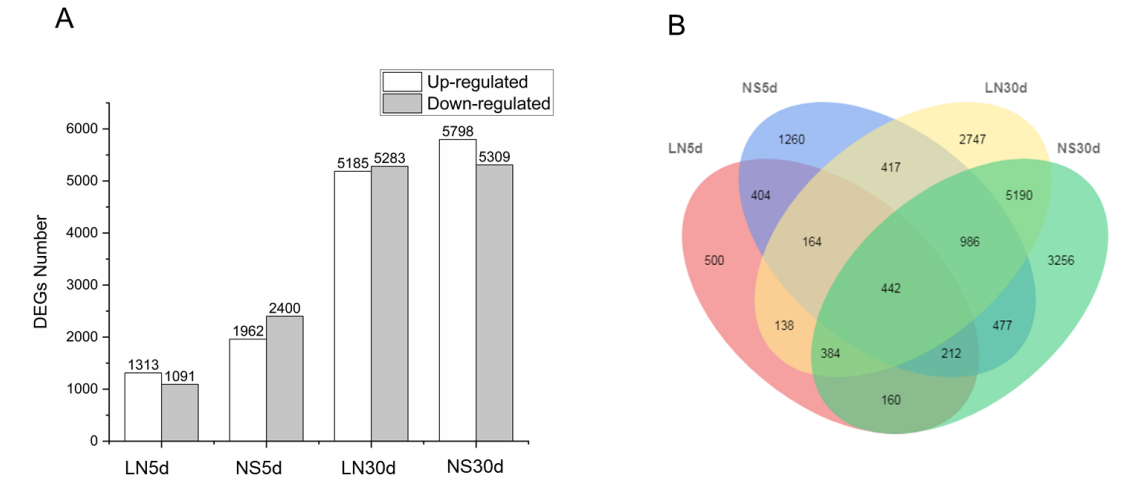

Figure 1. *Number of DEGs in different groups. (A) Up- and down-regulated DEGs after 5 and 30 days of treatment. (B) Venn diagram shows common DEGs among different treatment groups. LN5d and LN30d represent quinoa seedlings under Low nitrogen conditions for 5 and 30 days, respectively. NS5d and NS30d represent quinoa seedlings under nitrogen starvation conditions for 5 and 30 days, respectively. DEGs: Differentially expressed genes*

GO and KEGG analysis of DEGs

To clarify the functional distribution of DEGs, we used clusterProfiler to analyze DEG enrichment in the following GO categories: cell components, molecular functions, and biological processes. At 5 d, there were 872 and 1626 DEGs from the LN and NS treatment groups, respectively, enriched in GO terms. The most enriched terms were "cells, cell membranes and cell parts" in the cell components category, "catalytic activity, binding and transporter activity" in the molecular functions category, and "metabolic processes, cellular processes and single organism process" in the biological processes category (*Figure 2A*, *Table S3*). At 30 d, there were 4282 and 4534 DEGs from the LN and NS treatment groups, respectively, enriched in GO terms. The enrichment profile was similar to that at 5 d (*Figure 2B*, *Table S3*).

To further understand the metabolic pathways responding to different nitrogen treatments during the development of quinoa, we analyzed the 21 most enriched KEGG pathways of DEGs from day 5 samples in the LN and NS treatment groups. Among them, antioxidant biosynthesis was the most prominent category as it included phenylpropanol biosynthesis (ko00940), glutathione metabolism (ko00480), plant signal transduction (ko04075), plant–pathogen interaction (ko04626), ascorbic acid and aldehyde acid metabolism (ko00053), and alpha–linolenic acid metabolism (ko00592) (*Figure 3A*, *Table S4*). However, at 30 d, in the LN and NS treatment groups, photosynthesis and carbon metabolism were enriched, including carbon metabolism (ko01200), amino acid biosynthesis (ko01230), phenylpropanol biosynthesis (ko00940), carbon fixation in photosynthetic organisms (ko00710), photosynthesis (ko00195), glyoxylic acid and dicarboxylic acid metabolism (ko00630), and pyruvate metabolism (ko00620) (*Figure 3B*, *Table S5*).

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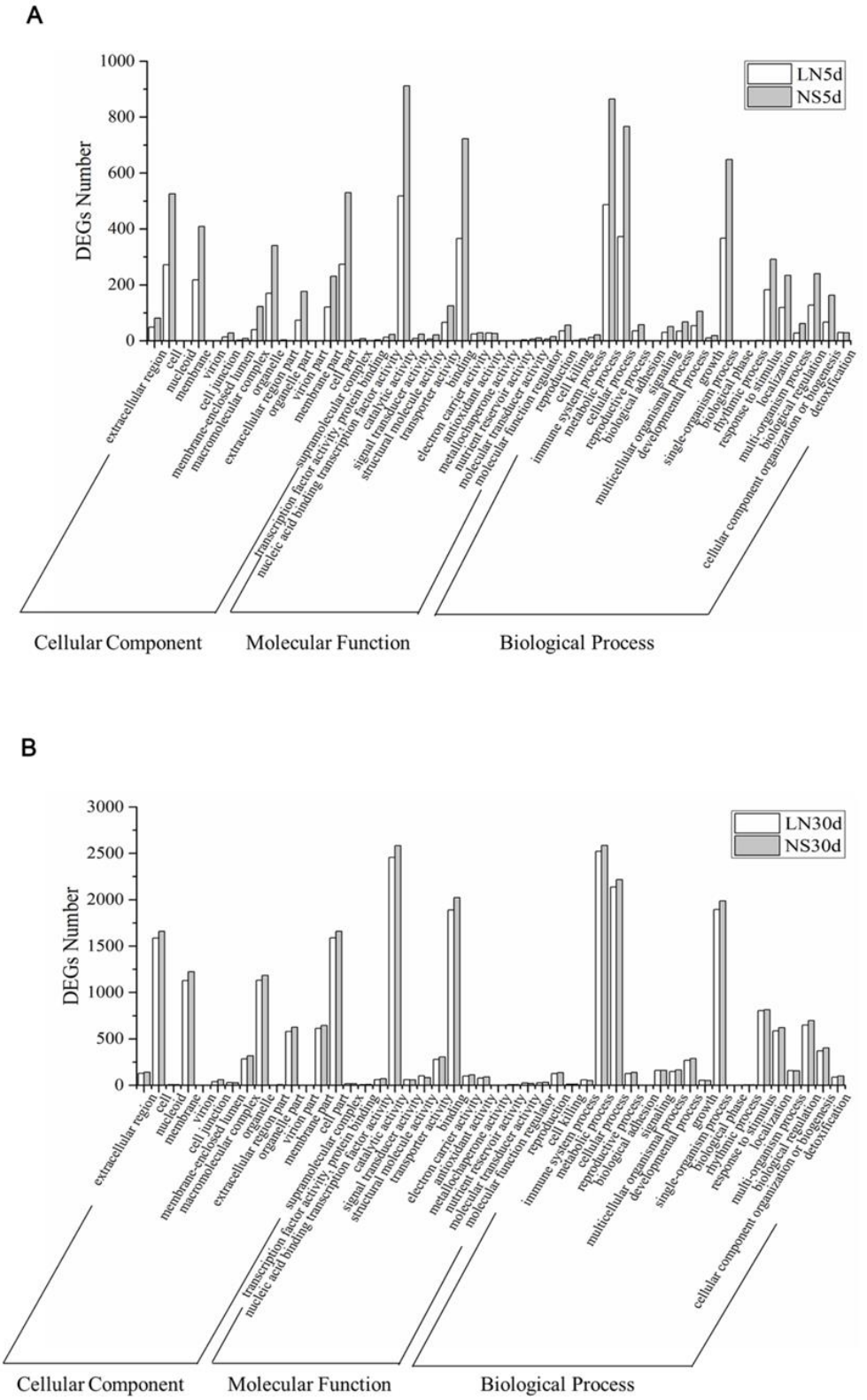

Figure 2. *GO classification of DEGs in different treatment groups. (A) DEGs in LN and NS treatment groups at day 5. (B) DEGs in LN and NS treatment groups at 30 days. LN5d and LN30d represent quinoa seedlings under low nitrogen conditions for 5 and 30 days, respectively. NS5d and NS30d represent quinoa seedlings under nitrogen starvation conditions for 5 and 30 days, respectively. DEGs: Differentially expressed genes*

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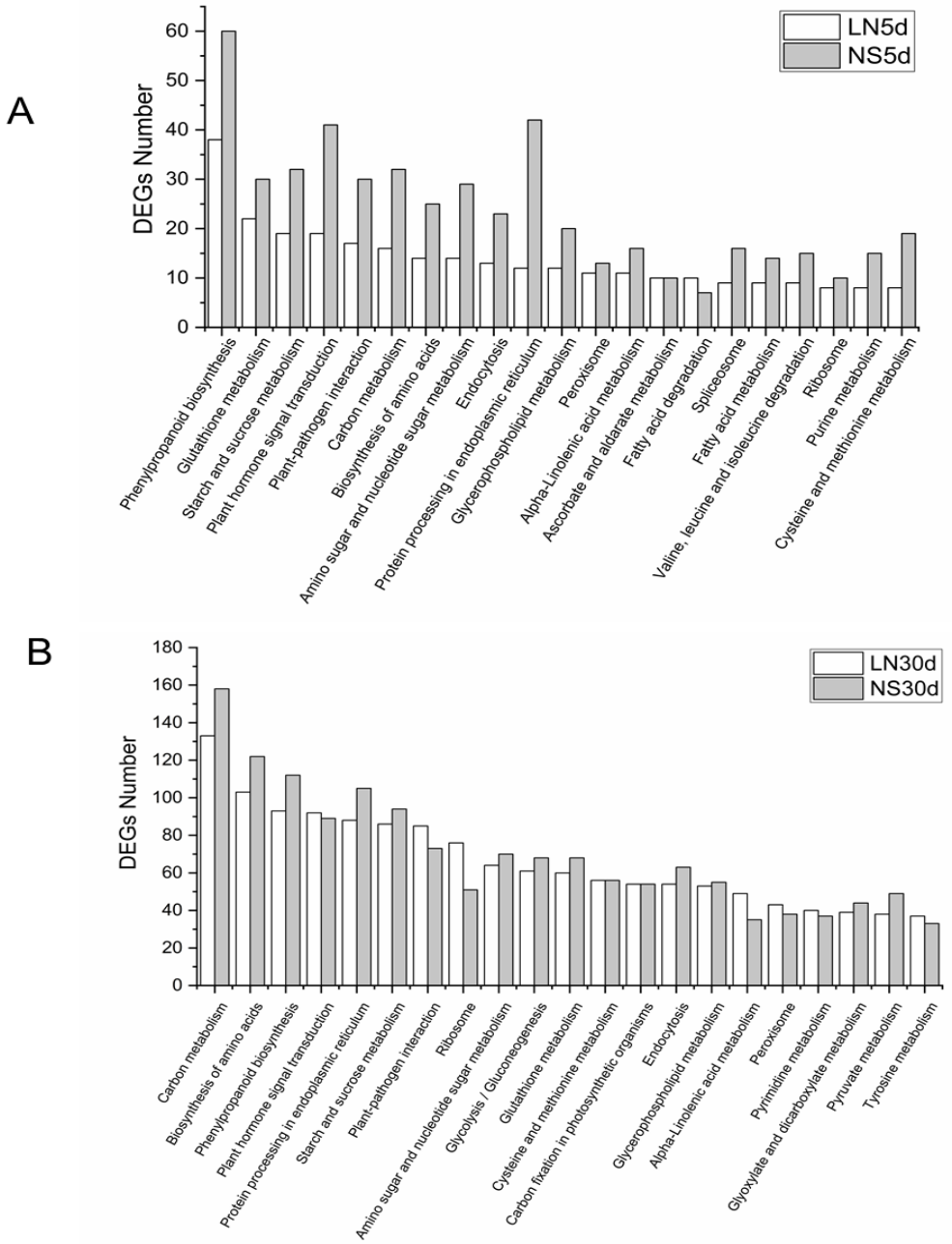

Figure 3. *KEGG pathway enrichment of DEGs. (A) KEGG pathway enrichment of DEGs in LN5d and NS5d. (B) KEGG pathway enrichment of DEGs in LN30d and NS30d. LN5d and LN30d represent quinoa seedlings under low nitrogen conditions for 5 and 30 days, respectively. NS5d and NS30d represent quinoa seedlings under nitrogen starvation conditions for 5 and 30 days, respectively. DEGs: Differentially expressed genes*

Genes involved in antioxidant biosynthesis and photosynthesis

We then analyzed the expression of genes related to the four key pathways involved in antioxidant systems and photosynthetic metabolism using heat maps. At day 5, genes related to phenylpropanoid biosynthesis (ko00940), glutathione metabolism (ko00480), plant hormone signal transduction (ko04075), and flavonoid biosynthesis (ko00941) had higher expression levels in the LN and NS treatment groups compared to those in the CK

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group. In addition, genes related to glutathione metabolism (ko00480) had a lower expression in the NS than in the LN treatment group, whereas genes related to phenylpropanoid biosynthesis (ko00940), plant hormone signal transduction (ko04075), and flavonoid biosynthesis (ko00941) had a higher expression in the NS than in the LN treatment group (*Figure 4A*, *Table S6*). These results indicate that nitrogen deficiency induces activity of antioxidant-related genes. At day 30, the expression levels of genes related to carbon metabolism (ko01200), pyruvate metabolism (ko00620), carbon fixation in photosynthetic organisms (ko00710), and glyoxylate and dicarboxylate metabolism (ko00630) were higher in the LN and NS treatment groups than in the CK group (*Figure 4B*, *Table S7*). These results suggest that genes related to photosynthesis especially to carbon assimilation was strongly responsive after 30 d of nitrogen deficiency.

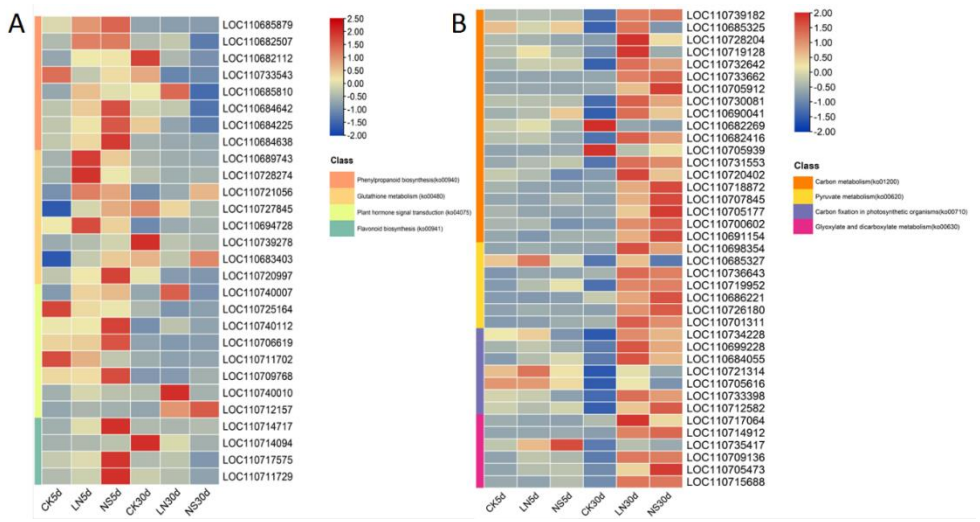

Figure 4. *Heat map showing DEGs involved in four key pathways related to antioxidant systems and photosynthetic metabolism in quinoa. (A) DEGs involved in the antioxidant system. Different colors represent phenylpropanoid biosynthesis (ko00940), glutathione metabolism (ko00480), plant hormone signal transduction (ko04075), and flavonoid biosynthesis (ko00941). (B) DEGs involved in photosynthetic metabolism. Different colors represent carbon metabolism (ko01200), pyruvate metabolism (ko00620), carbon fixation in photosynthetic organisms (ko00710), and glyoxylate and dicarboxylate metabolism (ko00630). LN5d and LN30d represent quinoa seedlings under low nitrogen conditions for 5 and 30 days, respectively. NS5d and NS30d represent quinoa seedlings under nitrogen starvation conditions for 5 and 30 days, respectively*

Expression profile of genes associated with photosynthesis

Photosynthesis is closely connected with biomass accumulation, and so we analyzed several candidate genes involved in photosynthesis. The Psb protein family is crucial for photosystem function. We identified five *Psb* genes in quinoa. At day 5, the expression level of one gene was higher (LOC110728024), and those of four genes were lower (LOC110710068, LOC110695639, LOC110704019, and LOC110689703) in the LN treatment group compared with in the CK group, whereas all five genes had lower expression in the NS treatment group compared with in the CK group. The expression levels of these five genes were higher in the LN and NS treatment groups at day 30 than

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at day 5, and their expression levels were higher in the LN treatment group than in the CK and NS groups at day 30. Of these five genes, LOC110710068 and LOC110728024 showed the highest expression levels in LN treatment group (*Figure 5A*, *Table S8*).

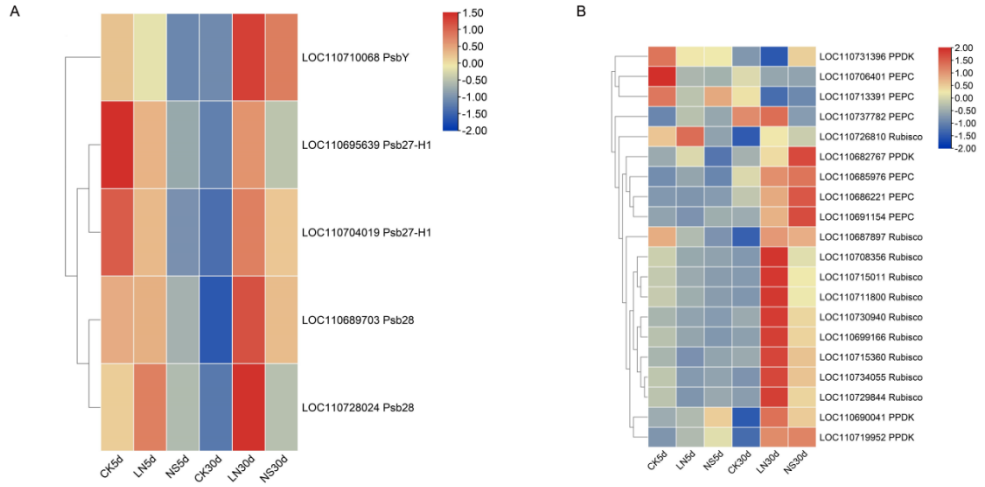

Figure 5. *Heat map of DEGs related to Psb family proteins and photosynthetic enzymes in quinoa. (A) DEGs related to Psb family proteins. (B) DEGs related to PPDK, PEPC, and Rubisco in quinoa. LN5d and LN30d represent quinoa seedlings under low nitrogen conditions for 5 and 30 days, respectively. NS5d and NS30d represent quinoa seedlings under nitrogen starvation conditions for 5 and 30 days, respectively*

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), phosphoenolpyruvate carboxylase (PEPC), and pyruvate, phosphate dikinase (PPDK) are three important enzymes involved in carbon assimilation process. Four *PPDK*, six *PEPC* and ten *Rubisco* genes were identified. At day 5, two *PEPC* and one *PPDK* genes were higher expressed in the CK group compared with those in the LN and NS treatment group. However, at day 30, ten *Rubisco* genes were all highly expressed in LN and NS groups compared with those in CK group, and this was especially true in the LN treatment group. Four *PEPC* genes were highly expressed in LN compared those in the CK, and three of them presented higher expression levels in NS treatment group (*Figure 5B*, *Table S9*). The qRT-PCR findings for six genes aligned with the transcriptome sequencing results (Supplementary 1). Given the remarkably higher expression levels of Rubisco and PEPC 30 days after treatment, we further compared their activities across the three treatment groups (*Figure 6*). The results were consistent with the gene expression profiles.

Gene expression level related to nitrogen metabolism

We further analyzed several genes closely connected with nitrogen metabolism, including nitrate reductase (NR), nitrite reductase (NiR), glutamine synthetase (GS), glutamine oxoglutarate aminotransferase (GOGAT), glutamate dehydrogenase (GDH), carbonic anhydrase (CA) and nitrogen transporter (NRT). In general, at day 30, more genes were highly expressed than at day 5 in LN and NS treatment groups. The expression levels of NR, NiR, and GS genes were higher, and those of GOGAT and GDH genes were lower in the LN and NS treatment groups compared with those in the CK group. The expression level of a nitrogen transporter gene, NRT2.5, exhibited no significant

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difference between the CK and LN treatment groups, but was higher in the NS treatment group compared with that in the CK group. Interestingly, within seven *CA* genes identified, five were higher expressed in LN and NS treatments compared with those in the CK group, and two uncharacterized genes (LOC110682416, LOC110717064) also showed higher expression level than the controls (*Figure 7, Table S10*).

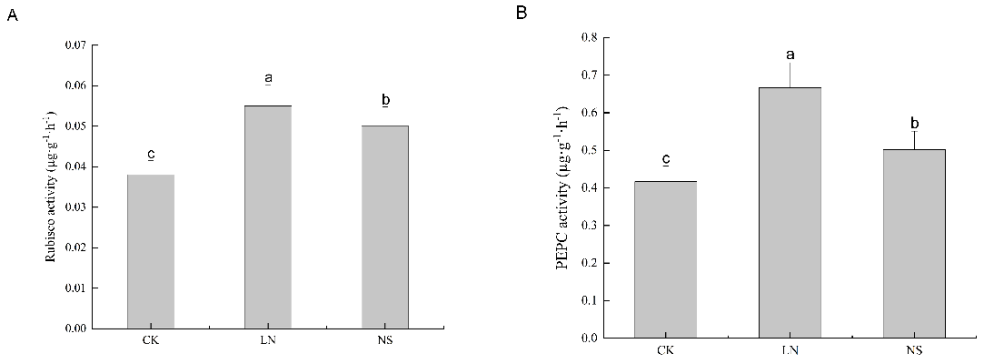

Figure 6. *Activities of Rubisco and PEPC. (A) Rubisco activity of quinoa seedlings 30-day post treatment. (B) PEPC activity of quinoa seedlings after 30 days treatment. LN and NS represent quinoa seedlings under low nitrogen and starvation conditions, respectively. *Differences among treatments were analyzed by one-way ANOVA, Duncan's multiple range test (n=3), different letters indicate significant difference at p<0.05*

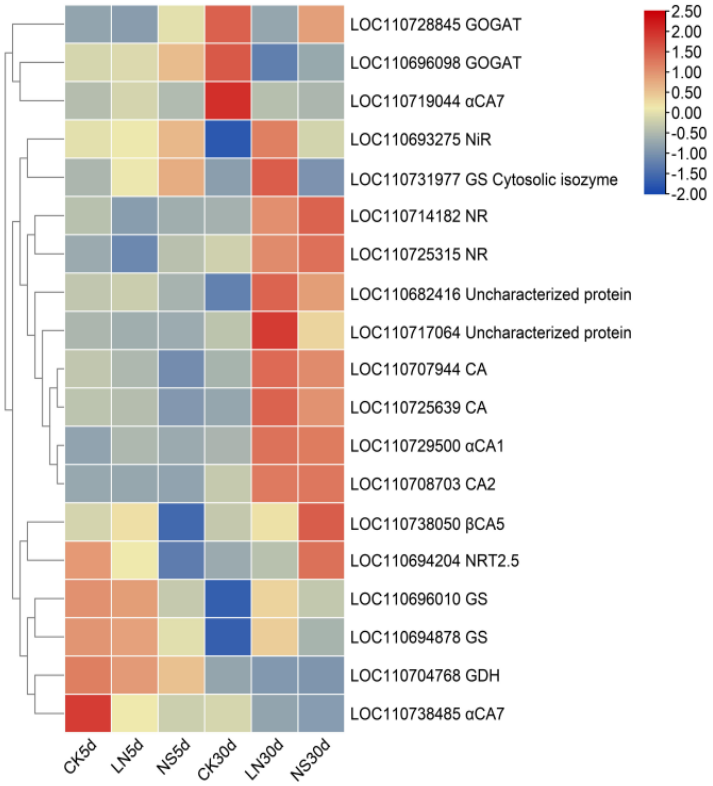

Figure 7. *Heat map showing DEGs involved in nitrogen metabolism pathways of quinoa. LN5d and LN30d represent quinoa seedlings under low nitrogen conditions for 5 and 30 days, respectively. NS5d and NS30d represent quinoa seedlings under nitrogen starvation conditions for 5 and 30 days, respectively. DEGs: Differentially expressed genes*

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Nitrogen deficiency changes photosynthetic parameters

RNA-seq analysis showed that after 30 d of nitrogen deficiency treatment, the most enriched KEGG pathways were related to photosynthesis. We then further measured the chlorophyll content and photosynthetic parameters. Leaf chlorophyll content was significantly lower ($p < 0.05$) in the LN and NS treatment groups compared with that in the CK group (*Table 2*). As expected, the photosynthetic parameters in LN and NS treatment were decreased compared with those in the CK, and the LN treatment had a smaller effect on photosynthetic parameters than the NS treatment.

Table 2. *Chlorophyll content and photosynthesis parameters of quinoa after 30 d of treatment (n=3 biologically independent samples)*

	Chlorophyll content ($mg \cdot g^{-1}FW$)	Pn ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$)	Fv/Fm	Yield	qP	qN
CK	1.80 ±0.1 a	2.05 ±0.19 a	0.78 ±0.07 a	0.671 ±0.06 a	0.94 ±0.09 a	0.501 ±0.05 a
LN	1.76 ±0.08 ab	1.82 ±0.15 a	0.69 ±0.06 b	0.581 ±0.05 a	0.93 ±0.08 a	0.285 ±0.02 b
NS	1.64 ±0.07 c	0.73 ±0.07 b	0.54 ±0.05 c	0.298 ±0.02 c	0.85 ±0.07 c	0.218 ±0.01c

*CK: Complete nutrient solution; LN: Low nitrogen solution; NS:Nitrogen starvation solution. Pn: Net photosynthetic rate; Fv/Fm: maximum photochemical efficiency of PSII; qP: photochemical quenching; qN: non-photochemical quenching. Different letters indicate significant differences at the 0.05 level. *Differences among treatments were analyzed by one-way ANOVA, Duncan's multiple range test, different letters indicate significant difference at $p<0.05$

Biomass accumulation under nitrogen deficiency conditions

After 30 d, seedlings in LN treatment group showed a favorable growth profile in that the plant height and biomass were the highest. After 120 d, the plants in CK group had the highest plant height and biomass, and those in NS group had the lowest. The lowest root weight showed in LN treatment, while the aboveground weight in LN treatment was the same as the CK. The root/shoot ratios further confirmed that under LN treatment, aboveground grow more preferentially, and root growth was hampered. With 20% nitrogen applied of the CK, grain yield and nitrogen concentration in LN treatment group was 67.4% and 58.2% of those in the CK, respectively (*Table 3*). We also found that at 120 d, grains were distributed in several panic in CK group, while they were more concentrated in LN treatment group. The stems in LN and NS treatment groups turned red, while those in the CK were still green (*Figure 8*).

Table 3. *Growth profile and grain yield (n = 10 biologically independent samples)*

Treatment	Plant height （cm）	Aboveground weight (g)	Underground weight (g)	Root-shoot ratio	Grain yield ($g\ plant^{-1}$)	Nitrogen content in seeds ($g.kg^{-1}$)
CK30	18.82 b	2.71 b	0.46 b	0.17 a	NA	NA
LN30	23.48 a	3.23 a	0.62 a	0.19 a	NA	NA
NS30	20.78 b	2.54 b	0.49 b	0.19 a	NA	NA
CK120	30.82 a	2.83 a	2.56 a	0.90 a	4.51a	3.95 a
LN120	24.60 b	2.83 a	0.73 c	0.26 c	3.04 b	2.30 b
NS120	23.38 b	1.93 b	1.11 b	0.58 b	0.93 c	2.25 b

*CK30, LN30 and NS30 represent quinoa seedlings under CK, LN and NS conditions for 30 d, respectively; CK120, LN120 and NS120 represent quinoa plants under CK, LN and NS conditions for 120 d, respectively. *Differences among treatments were analyzed by one-way ANOVA, Duncan's multiple range test, different letters indicate significant difference at $p<0.05$

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Figure 8. *Quinoa plant profile after 120 d of treatment and was altered after different nitrogen treatments. Top row showing plant growth profile in CK, LN and NS treatment groups. Bottom row showing tassels profile in in CK, LN and NS treatment groups*

Discussion

As a nutrient-rich plant of the Amaranthaceae family, quinoa is considered a promising alternative crop, especially for developing countries, to maintain food security (Jancurova et al., 2009; Suttiarporn et al., 2015; Bascuñán et al., 2018). Though many genes linked to nitrate availability have been identified in many plant species, such as *Arabidopsis thaliana*, maize (*Zea mays*) and wheat (*Triticum aestivum*) etc. (Guan et al., 2017; Swift et al., 2020; Meng et al., 2020), these genes have not yet to be studied in quinoa.

When quinoa was subjected to LN and NS treatments for 5 days, the metabolic systems related to antioxidants were the most enriched pathways. In wheat, genes related to antioxidant enzymes were up-regulated under low-nitrogen conditions (Wang et al., 2019), and this is also true for rice and Arabidopsis when under low nitrogen stress. Herein, up-regulated phenylpropanoid and flavonoid biosynthesis in quinoa seedlings after LN and NS treatment might be an adaptive mechanism to environmental stress induced by nitrogen shortage.

After 30 d of LN and NS treatments, photosynthesis related pathways were highly enriched. Nitrogen deficiency hamper the chlorophyll biosynthesis, therefore, the chlorophyll content and chlorophyll fluorescence parameters were reduced in LN and NS treatment groups. However, the expression level of *Psb*-related genes were up-regulated in the LN treatment group. Psb family proteins are crucial photosystem members in plants and they are the key components of the cytochrome b6/f complex, photosynthetic electron transport, and photosystem II (Kawaguchi et al., 1992; Yi et al., 2008; Króliczewski et al., 2017; Kato et al., 2017; Rikard et al., 2017). Previous studies have shown that the PsbS protein plays a central role in protecting the photosystem from strong light damage (Correa et al., 2016). In wheat, *Psb* genes were down-regulated under nitrogen-deficient conditions (Liu et al., 2020) which is in contrary to the present study. Herein the up-

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regulated *Psb*-related genes might partially alleviate the photosynthesis inhibition induced by nitrogen shortage. Rubisco is the most abundant enzyme involved in carbon assimilation. Here, all 10 identified *Rubisco* genes were highly triggered, this is especially true in LN treatment group, which could explain the higher biomass in LN plants at 30 d. We further compared gene copy number of Rubisco with some species and found the greater number in quinoa than those in all the examined plant species including soybean, potato, and cereal crops as maize and rice (*Supplementary 2*).

Within genes involved in nitrogen metabolism, the *CA* genes were the most obviously up-regulated ones. Four and five genes showed higher expression level in LN and NS treatment groups, respectively. The function of *CA* genes in photosynthesis has been well documented (Badger et al., 2003; Naeem et al., 2009); and today its non-photosynthetic role has been attracted attention. In *Lotus japonicus*, the nodule-enhanced expression patterns and the diverse distributions of *CA* genes imply their potential functions like N assimilation (Wang et al., 2021). Here, *CA* genes strongly responded to low nitrogen application in quinoa which may be an interesting future topic to explore. Nitrogen assimilatory enzymes including NR, NiR, GS, GOGAT and GDH have long been suggested to play important roles in governing the NUE of crops (Garnett et al., 2009), and NR was proposed as the rate limiting enzyme involved in nitrate assimilation. Here in the LN treatments, those enzymes did not exhibit exceptional performance. NR showed higher expression level, while the rest enzymes presented lower expression level. However, efforts on breeding plants with higher NR activity or over-expression of NR were having little impact on the NUE of crops (Good et al., 2004; Garnett et al., 2009). Swift et al. (2020) demonstrated that in Arabidopsis, NRT1.1 is in the pathway that control rates of transcriptional change in response to N-dose. Herein, one NRT gene was identified involving in nitrogen metabolism upon varied nitrogen supply, but its expression level was not different with the control.

We also observed a change in stem color under nitrogen deficiency conditions, with red stems appeared in both LN and NS groups. Anthocyanins, known for their role as stress protectants induced by various biotic and abiotic stress conditions, including nitrogen depletion (Saigo et al., 2020), presented an interesting facet. In Arabidopsis, anthocyanins represent a metabolic marker of nitrogen deficiency (Nunes-Nesi et al., 2010; Watanabe et al., 2013). Similarly, an increase in anthocyanin accumulation under low nitrogen conditions has been observed in other plants such as apple (*Malus pumila* Mill.), lettuce *(Lactuca sativa* L.) and *Malus spectabilis* (Becker et al., 2015; Sun et al., 2018; Meng et al., 2023). The results of our study are in agreement with those observations.

Conclusions

The results provide insight into the molecular and physiological mechanisms associated with the low-nitrogen adaptations of quinoa. Under nitrogen deficiency conditions, chlorophyll synthesis was hampered. However, the up-regulated *Psb*-related genes might partially alleviate the photosynthesis inhibition. All 10 identified *Rubisco* genes were highly triggered and these could explain the higher biomass in LN plants 30 days after treatment. We therefore propose that quinoa might possess exceptional carbon assimilation ability. The exceptional higher copy numbers of *Rubisco* genes and their actively responsive response to nitrogen deficiency conditions might be a molecular basis accounting for the high carbon assimilation capacity of quinoa. In nitrogen

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metabolism, *CA* genes were strongly responsive to nitrogen deficiency. Herein, we identified several genes actively involved in quinoa responses in low nitrogen conditions at RNA level. The results could be useful for breeding crop cultivars that tolerate nitrogen deficiency. However, what the detailed performance of these genes and their biological functions will ultimately require further studies.

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APPENDIX

This manuscript has electronic appendices.

Supplementary note 1. Quantitative real-time PCR validation of RNA-Seq data

RNA extraction and real-time qPCR analysis

Candidate differentially expressed genes were validated by qRT-PCR analysis using three biological replicates. Gene-specific primers (*Table S1*) were designed using Primer 5.0 and synthesized commercially (Sangon Biotech Co. Ltd, Shanghai, China). First-strand cDNA was synthesized from 0.8 μg of total RNA using Revert Aid Premium Reverse Transcriptase (Thermo) in accordance with the manufacturer's instructions and reverse transcribed into cDNA followed by 10× dilution. The primer efficiency tests were done for qPCR primers. qRT-PCR was performed in a 20 μL reaction volume containing 2 μL of 10× diluted cDNA as the template, 0.4 μL of each 10 μM forward and reverse gene specific primer. The qRT-PCR reactions were performed on an ABI Stepone plus real-time PCR system (Applied Biosystems, Foster City, CA, USA) using SG Fast qPCR Master Mix (High Rox). Relative quantification of gene expression was calculated and normalized using CqEF-1a as an internal standard. The comparative Ct ($2^{-(\Delta\Delta Ct)}$) method was used to calculate the fold-changes in gene expression level.

Table S1. *Genes verified by qRT-PCR and their primer sequences*

Gene Name	Gene Annotation	Primer sequence (5′→3′)
CqEF-1a (actin)	-	F: GTACGCATGGGTGCTTGACAAACTC R: ATCAGCCTGGGAGGTACCAGTAAT
AUR62004699	Nitrate reductase	F: GCAGAGGAGGACATATTG R: TCTCAACAACATACCATACTT
AUR62029383	Nitrite reductase	F: ATAAGGATGATGTTGATGTTAGA R: TTGCTCACTCGTTGTTAC
AUR62041208	Glutamine synthetase	F: CAAGCACCAGGAGAAGAC R: TGTTACCACCACGGAATG
AUR62042896	Phosphoenolpyruvate carboxylase	F: TGGTGGTGGATATGAAGA R: GACAGATTGAGTAGGATGAG
AUR62038255	MYB44	F: TGTAGTAGTAGCGAGAATA R: CTTGTTAAGGCATCATCT
AUR62043570	Uroporphyrinogen decarboxylase	F:TGCCCTGAAACACCACTTG R:CAGTCCAGCCCAATCACATT

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Figure S1 shows a list of genes that are up- or down-regulated at 5 d post LN and NS treatment, and a comparison with the results obtained by Fluorescent quantitative reverse transcription PCR. The fold changes of these genes obtained from qRT-PCR assays were compared with RNA-Seq results which validated the accuracy and robustness of the RNA-Seq results.

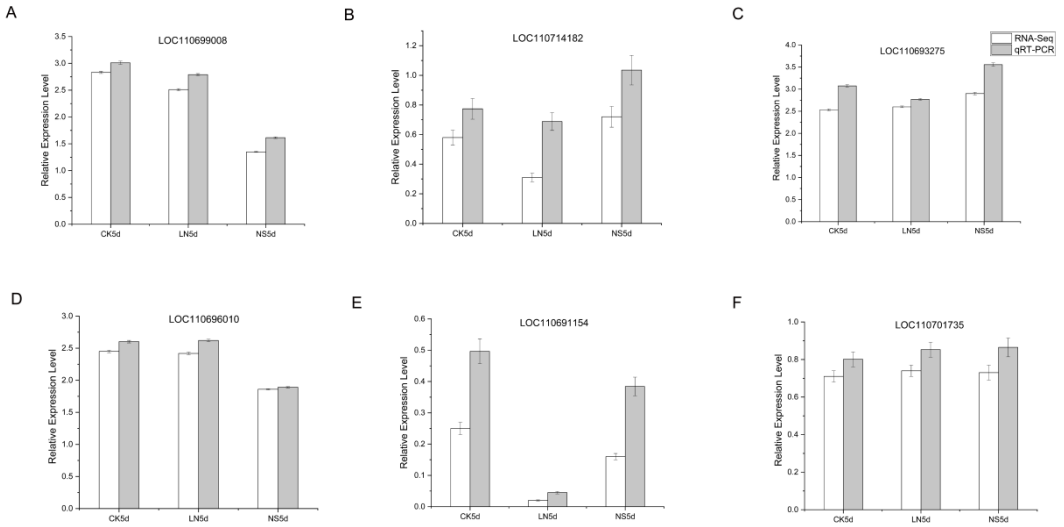

Figure S1. *RNA-seq results were verified by qRT-PCR. LOC110714182: nitrate reductase (NR); LOC110696010: glutamine synthase (GS); LOC110693275: nitrite reductase (NiR); LOC110691154: phosphoenolpyruvate carboxylase (PEPC); LOC110701735: MYB44; LOC110699008: uroporphyrin decarboxylase (UROD)*

Supplementary note 2. Copy number of Rubisco

A well-characterized Arabidopsis member of Rubisco family was used as a seed to search for similar protein sequences in quinoa and other plant species using BLASTP. Candidates with an E-value smaller than $10e^{-5}$ were then searched against the Pfam (http://pfam.xfam.org/search) to ensure that all the major conserved domains that are present in the seed are also present in the candidates. The candidate protein sequences were then manually examined to exclude the outliers that have very short protein sequences relative to others. The gene copy number in each species was calculated manually.

Table S2. *Copy number of Rubisco in selected plant species*

	At	Gm	Zm	St	Bv	Os	Cq
Rubisco	8	2	2	4	5	5	9

Species are indicated by two-letter names: At, *Arabidopsis thaliana*; Gm, *Glycine max*; Zm, *Zea mays*; St, *Solanum tuberosum*;Bv, *Beta vulgaris*; Os, *Oryza sativa*; Cq, *Chenopodium* quinoa

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