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Transcriptomic profiling of shallots (*Allium cepa* var. *aggregatum*) reveals differential gene expression patterns under nitrogen deficiency

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Abstract: Nitrogen (N) is essential for crop productivity, yet excessive fertilisation decreases the nitrogen use efficiency and increases the environmental risks. Despite the economic and medicinal importance of the shallot, its molecular response to N deficiency remains unclear. We performed transcriptome profiling of two cultivars (Bima-0 and Bima 1) under N deficient and N-sufficient conditions. The RNA sequencing identified 438 differentially expressed genes (DEGs). The gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis revealed significant modulation of the nitrogen metabolism, amino acid biosynthesis, glutathione metabolism, and stress responses pathways, indicating coordinated metabolic reprogramming under N limitation. The cultivar-specific expression patterns suggested differential adaptive strategies. The quantitative real-time polymerase chain reaction (qRT-PCR) validation of fifteen DEGs confirmed the reliability of the RNA-Seq results. The nitrogen deficiency predominantly repressed the photosynthetic and growth-linked genes while activating the stress-responsive pathways. These findings provide the first transcriptome-level insights into the N deficiency responses in shallots and identify candidate genes and pathways for improving the nitrogen use efficiency (NUE) and developing the sustainable fertiliser management of *Allium* crops.

Keywords: DEGs; gene enrichment; gene expression; metabolic pathways; nitrogen use efficiency

Allium cepa var. *aggregatum*, (shallot) is an economically important vegetable valued for its nutritional, medicinal and commercial significance (Mahmood et al. 2021). The demand continues to rise domestically and internationally, increasing the export volume and market relevance. Shallots are widely used as a culinary spice (Kuete 2017), natural preservative, rich antioxidant source (Mardani et al.

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2023) and traditional medicine ingredient (Banerjee et al. 2022). Shallots are cultivated across diverse agro-ecological zones, mainly in dryland tropical regions where productivity is greatly influenced by the limited water supply, high temperatures, low humidity, heavy and unpredicted rainfalls and fog (Assefa et al. 2016; Xie et al. 2020).

In Indonesia, shallot production increased from 1 048 934 tonnes in 2010 to 1 815 445 tonnes in 2020, yet productivity declined from 10.2 t/ha (2013) to 9.7 t/ha (2020), despite the expansion of the cultivated area (FAO 2022). The lower productivity is associated with mono-cropping, short rotations, suboptimal management, soil-borne diseases and, particularly, nutrient limitation. Nitrogen (N) is the most critical macronutrient, essential for enzymes, amino acids, chlorophyll and overall plant growth (Brady 1984).

Although nitrogen fertilisation has significantly increased global crop yields, nitrogen fertiliser applications are projected to reach 135 teragrams per year by 2050 (Good et al. 2004), raising concerns about soil degradation, environmental pollution and production costs. Enhancing the nitrogen use efficiency (NUE) is therefore essential to maintain yields under low nitrogen conditions while minimising the environmental risks. The NUE depends on the efficient uptake, assimilation and remobilisation of nitrogen within plants and remains a major focus of agricultural research (Masclaux-Daubresse et al. 2010).

The onion *(Allium cepa)* possesses a large diploid genome (> 16.4 gigabases (GBs); $2n = 16$), among the largest known for diploid plants (McCallum et al. 2012). Despite cytogenetic studies, molecular analyses remain limited. The shallot lacks a reference genome, restricting the molecular understanding of the nutrient response. Although proteomic studies have examined abiotic stress in *Allium* species (Chen et al. 2013; Qin et al. 2016), a transcriptome-scale analysis of the nitrogen deficiency in shallots has not been reported on, creating a significant knowledge gap.

Next-generation sequencing (NGS) provides a cost-effective platform for transcriptomic analysis, enables the identification of differentially expressed genes even without a reference genome. This approach has been widely applied to economical important crops to characterise the stress responsive and metabolic pathways (Rashmi et al. 2019). High throughput sequencing technologies, including the Illumina platform, have facilitated accurate and large-scale transcriptome profiling (Mine et al. 2014). In this context, the present study provides the first transcriptome-level investigation of nitrogen deficiency in shallots, generating fundamental insights into N responsive genes and pathways in this economical important yet genetically understudied crop.

MATERIAL AND METHODS

Plant material, treatment and sampling. Two shallot cultivars, Bima-0 and Bima-1, were evaluated under two nitrogen treatments: (*i*) N-control (recommended dose) and (*ii*) N-stress (no nitrogen). The recommended rate (115 N kg/ha; equivalent to 250 kg urea/ha, 46% N), was proportionally converted to 4.5 g urea per polybag (three plants per bag) and applied in two equal splits (2.25 g each). To impose the whole plant nitrogen deficiency, the seedlings were grown in a nitrogen free medium from germination until sampling, and no nitrogen was applied to the N-stressed plants at any stage. All the other nutrients were applied uniformly to avoid cofounding deficiencies. The experiment was arranged in a split block design with three replications and spacing of 10 × 20 cm (plant-to-row).

For the RNA sequencing (RNA-Seq), surface-sterilised seedlings were grown in a growth chamber at 26/24 °C (day/night) and 75% relative humidity, with three biological replications per treatment. The RNA-Seq analysis was performed using three biological replicates per treatment and technical replicates were not included as biological replication is considered sufficient for a robust differential gene expression analysis. Twenty-one days after germination, urea was applied to the N-control plants, while the N-stressed plants continued without any nitrogen supply. Leaf samples were collected twenty-four hours after the urea application from both treatments within each cultivar. A differential expression analysis was performed separately for each cultivar. However, nitrogen stress is the first perceived in roots, the leaves were selected for the analysis as the primary site for the nitrogen assimilation and photosynthetic regulation. No direct comparison was made than between the cultivars (Bima-0 and Bima-1). The samples were immediately frozen in liquid nitrogen and stored at −80 °C until further processing.

RNA extraction and reverse transcription. The total RNA was extracted from the leaf samples using a Quick-RNA Miniprep Kit (Zymo Research, USA) according to the manufacturer's instructions and further purified using an RNeasy Plant Mini Kit (Qiagen, Germany). The RNA concentration and purity were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) and the integrity was verified on a 1.2% agarose gel. The first-strand complementary DNA (cDNA) was synthesised from one microgram of RNA using a Verso cDNA Kit (Thermo Fisher Scientific, USA) with oligo$(dT)_{18}$ primers. The synthesised cDNA was stored at –20 °C.

RNA-Seq library preparation, sequencing and quality assessment. The paired end libraries (150 bp) were prepared using a Collibri™ standard RNA library prep kit for Illumina™ Systems (Thermo Fisher Scientific, USA). The library quality and concentration were evaluated using Quantitative real-time polymerase chain reaction (qRT-PCR) and TapeStation analyses. Sequencing was performed on the Illumina NextSeq 500 platform (Illumina Inc., USA). The raw reads from each sample were initially assessed for quality using MultiQC (Ver. 1.13) (Huang et al. 2021). The adapter sequence and low-quality reads were removed using fastp (Ver. 0.23.2) (Chen et al. 2018). The quality of the filter reads was re-evaluated using FastQC (Ver. 0.11.9) (Brown et al. 2017). Only high-quality reads (Q20 > 98%, Q30 > 93%) were retained for the downstream analysis. To minimise bias due to the sequencing depth variation, filtered reads from each sample were subsampled to match the lowest read count among all the samples.

Due to the absence of a fully annotated reference genome for shallots, a *de novo* transcriptome assembly approach was employed. High-quality filtered reads from all the samples were combined and assembles using Trinity (Ver. 2.8.5) with a default parameter. The quality of the assembled transcriptome was evaluated using rnaQuast (Ver. 2.2.1), based on metrics such as the transcript length distribution and N50 values. The transcriptome completeness was further assessed using Benchmarking Universal Single-Copy Orthologs (BUSCO) (Ver. 5.4.4). The coding regions within the assembled transcripts were predicted using TransDecoder (Ver. 5.5.0) and any redundant sequences were removed using CD-HIT to generate a non-redundant transcript dataset.

Differential gene expression analysis. The differential expression analysis under nitrogen stress was performed using DESeq2 (Ver. 1.38.3), with a raw count matrix. Genes with low expression (total count less than 10) across replicates) were filtered prior to the analysis. Normalisation was conducted using the median-of-ratios approach implemented in DESeq2 where the fragments per kilobase of transcript per million mapped reads (FPKM) or transcripts per million (TPM) values were not used. Significantly differentially expressed genes (DEGs) were identified at a false discovery rate (FDR)-adjusted *P*-value (Benjamini & Hochberg 1995) threshold of < 0.05 and an absolute log2 fold-change cut-off of ≥ 1.

Functional annotation and gene ontology (GO) term analysis. GOSeq (Ver. 1.50.0) was utilised for the gene ontology enrichment analysis (Young et al. 2010). FASTA sequences of the up and down-regulated genes were retrieved from the assembled transcriptome generated by trinity and the functional annotation was performed using Trinotate (Ver. 3.2.2) under the biological process (BP), cellular component (CC) and molecular function (MF) categories.

The DEGs were functionally annotated using PFAM (HMMER Ver. 3.3.2), EggNOG (EggNOG Mapper Ver. 2.1.1), SwissProt (blastp; BLAST Ver. 2.13.0+), and SignalP (Ver. 4.1) through Trinotate (Ver. 3.2.2) (Camacho et al. 2009; Petersen et al. 2011; Eddy 2009; Cantalapiedra et al. 2021). Volcano plots were constructed using ggplot2 (Ver. 3.4.2) (Wickham 2011), and Venn diagrams were generated using Venny 2.1.

Enrichment analysis based on gene ontology terms. GO enrichment was conducted using GOSeq (1.50.0) to identify significantly enriched GO terms among the DEGs under N deficiency (Young et al. 2010). A false discovery rate (FDR)-adjusted *P*-value (Benjamini–Hochberg correction) of < 0.05 was used as the threshold for significant GO term enrichment.

KEGG pathway analysis. The Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses of all the DEGs were conducted using ClusterProfiler (Ver. 4.6.2) to identify the pathways linked with the nitrogen stress response (Yu et al. 2012).

Quantitative real-time PCR validation of RNA-Seq data. The RNA-Seq results were validated by qRT-PCR following the minimum information for publication of the quantitative real-time PCR experiments (MIQE) guidelines (Bustin et al. 2009). Fifteen DEGs were selected based on the high differential expression ($\log_2$FC ≥ 2), functional relevance to nitrogen metabolism, and representation of the up- and down-regulated genes. Gene-specific primers (Table S1 in Electronic Supplementary Material (ESM)) were developed using primer3 (Untergasser et al. 2012) with an amplicon size of 100 to 200 bp, melting temperature (Tm) of 58–62 °C, guanine and cytosine (GC) contents of 50–60%, and validated against the *Allium cepa* sequence. *Actin* was used as the reference gene (Sulistyaningsih et al. 2024).

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Using a volume of 20 μL, comprising 10 μL of a 2× PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, USA), 0.4 μL each of the forward and reverse primer (10 μM; final concentration 200 nM), 2 μL of the dilute cDNA template and 7.2 μL of nuclease free water, it was used for the qRT-PCR. The reaction composition and primer concentration were selected based on standard SYBR Green qPCR protocols and widely adopted practices in gene expression studies (Udvardi et al. 2008; Bustin et al. 2009). Amplification was conducted using the QuantStudio 5 Real-Time PCR system (Thermo Fisher Scientific, USA) with the following conditions; initial denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s.

All the reactions were conducted using three biological replicates, and each biological replicate was analysed with three technical replicates to ensure the accuracy and reproducibility of the expression data. The data were presented as the mean ± standard deviation (SD) and the relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. The statistical significance between the control and the nitrogen stressed plant was determined by a *t*-test.

RESULTS AND DISCUSSION

Sequencing and de novo assembly of the shallot leaf transcriptome. Illumina NextSeq500 generated 35.72 million raw paired-end reads (150 bp) from the leaf samples of both cultivars under nitrogen stress and sufficient conditions. After quality filtering, 34.25 million clean reads were retained (Q20 > 98% and Q30 > 93%) with stable GC contents and were used for the transcriptome assembly.

The high-quality data ensured the reliable transcriptome assembly and downstream analysis. Consistent with previous reports that the *Allium* species possess a large and complex genome that hindered genomic progress (Ricroch et al. 2005, McCallum et al. 2012), transcriptomic-based approaches remain essential for uncovering stress responsive pathways (Zhang et al. 2012).

Differential gene expression in response to nitrogen deficiency stress. The differential expression analysis was performed independently for each cultivar using DESeq2 (FDR < 0.05, $\log_2FC \geq 2$). In total, 438 DEGs were identified, including 286 down regulated and 152 up regulated (Figure 1), indicating transcriptional repression under nitrogen deficiency. This suggests a shift toward energy conservation by suppression of the growth and photosynthesis, a common adaptive response under nitrogen stress. Other down-regulated DEGs are presented in Table S2 in ESM. The volcano plot (Figure 2) displays a predominance of down-regulated genes, indicating strong transcriptional repression under nitrogen deficiency. In contrast, few genes showed strong up-regulation. A similar repression-dominated response has been reported in physic nuts (Kuang et al. 2017) and rice (Sinha et al. 2018), whereas the Welsh onion exhibited relative more up-regulation (Zhao et al. 2021), suggesting species specific nitrogen adaptation strategies.

The enrichment analysis revealed significant modulation photosynthesis, carbon fixation, carbohydrate metabolism and lipid related pathways, highlighting the strong coordination between the carbon and nitrogen metabolism. The nitrogen deficiency is also known to affect the carbon and nitrogen balance, and impair the photosynthetic metabolism (Duan et al. 2018; Naliwajski & Skłodowska 2018). Consistent with these reports, the transcriptional profile of the shallots suggests a conservative metabolic strategy prioritising survival and internal nitrogen conservation over active growth.

The top up- and down-regulated DEGs are listed in Table 1 and Table S3 in the ESM. Among the up-regulated genes, *UCH_1* showed the strongest

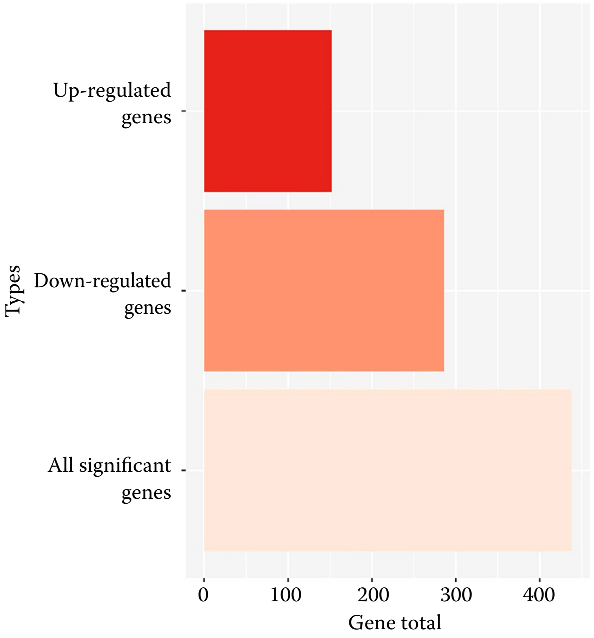

Figure 1. Number of up- and down-regulated differentially expressed genes (DEGs) expressed in nitrogen stress

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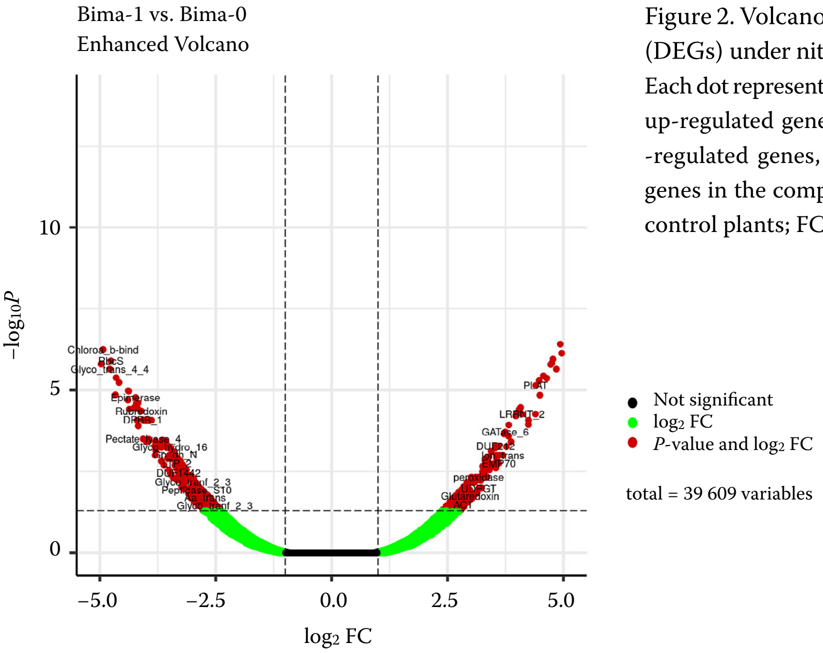

Figure 2. Volcano plot of the differentially expressed genes (DEGs) under nitrogen deficiency

Each dot represents a single gene; red dots indicate significantly up-regulated genes, green dots indicate significantly down-regulated genes, and black dots represent non-significant genes in the comparison between the nitrogen-stressed and control plants; FC – fold change

induction ($\log_2$FC = 4.24; FDR = 8.40 ×10^{-5}), followed by *PLAT*, *p450* and *LRRNT_2* (Table 1). The induction of *UCH_1* implies the activation of ubiquitin mediated protein degradation, which facilitates the nitrogen remobilisation and recycling (Masclaux-Daubresse & Chardon 2011). The up-regulation of *P450* and *PLAT* genes indicates stress linked metabolic adjustments, consistent with previous reports linking metabolic reprograming to abiotic stress responses (Singh et al. 2022). The induction of *LRRNT_2* suggests the involvement in stress signalling and transcriptional regulation, similar to observations in maize (Lu et al. 2021) and in the Welsh onion (Zhao et al. 2021).

The most strongly down-regulated gene was *RbcS* ($\log_2$FC = –5.96; FDR = 6.25 ×10^{-09}), along with *Pectate_lyase_4*, *LTP_2* and *Glyco_hydro_9* (Table 1). The down-regulation of *RbcS* suggests reduced photosynthetic capacity, as ribulose-1,5-bisphosphate

Table 1. Top up- and down-regulated differentially expressed genes (DEGs) in the nitrogen-stress plants compared to the control of the same shallot cultivars

Gene name	Putative protein (annotation)	Log$_2$FC	*P*-value	FDR
UCH_1	ubiquitin carboxyl-terminal hydrolase	4.24	1.31 × 10^{-07}	8.40 × 10^{-05}
PLAT	PLAT/LH2 domain	4.40	6.48 × 10^{-09}	7.13 × 10^{-06}
p450	cytochrome P450	3.62	2.61 × 10^{-06}	0.000997
LRRNT_2	leucine rich repeat N-terminal domain	4.10	7.63 × 10^{-08}	5.43 × 10^{-05}
Ion_trans	ion transport protein	3.69	2.61 × 10^{-06}	0.000997
GATase_6	glutamine amidotransferase domain	3.74	3.35 × 10^{-07}	0.0001925
EMP70	endomembrane protein 70	3.60	5.61 × 10^{-06}	0.001764
DUF212	divergent PAP2 family	3.53	1.18 × 10^{-06}	0.000525
Bet_v_1	pathogenesis-related protein Bet v 1 family	3.55	3.55 × 10^{-06}	0.001256
RbcS	ribulose-1,5-bisphosphate carboxylase small subunit	–5.96	6.36 × 10^{-13}	6.25 × 10^{-09}
Pectate_lyase_4	pectate lyase	–5.58	1.71 × 10^{-11}	5.49 × 10^{-08}
LTP_2	probable lipid transfer	–6.27	8.24 × 10^{-14}	1.09 × 10^{-09}
Glyco_hydro_9	glycosyl hydrolase family 9	–5.46	1.80 × 10^{-11}	5.49 × 10^{-08}

Positive $\log_2$FC indicates up-regulation in nitrogen stress relative to nitrogen efficient, whereas negative values indicate down-regulation; $\log_2$FC – $\log_2$ fold change; FDR– false discovery rate carboxylase/oxygenase (RuBisCO) is a nitrogen rich protein (Masclaux-Daubresse et al. 2010). This repression represents an adaptive mechanism to reduce the nitrogen demand and optimise the resource allocation under deficiency conditions. Similarly, the repression of photosynthetic genes has been reported in barley (Wei et al. 2016) and cotton (Iqbal et al. 2020), indicating a conserved mechanism to decrease the energy expenditure in nitrogen deficiency. The down-regulation of the Calvin cycle and glycolytic genes further suggest reducing the carbon flux toward growth; which aligns with the finding of Sultana et al. (2020) in wheat. The enrichment of glyoxylate and dicarboxylate metabolism indicates metabolic reprogramming to maintain the cellular energy balance (Mauceri et al. 2022). Overall, the nitrogen stress in shallots suppresses the growth linked processes, while the activation of nitrogen recycling and stress responsive pathways, reflects a coordinated strategy for adaptation to nutrient deficiency.

Gene ontology and KEGG enrichment analysis of DEGs. The GO analysis assigned 208 DEGs to the biological process category (Figure 3). The main enriched processes included the stress response (cold), photosynthesis and carbon fixation (dark reaction and reductive pentose-phosphate cycle), cell wall organisation and lipid metabolism; indicating metabolic and structural adjustment under nitrogen deficiency. The top ten biological process are presented in Table S4 in the ESM.

The GO enrichment for the cellular components identified 52 significantly enriched terms (Figure 4; Table S5 in the ESM), mainly linked with were the extracellular area (GO: 0005576; *P*-value of 2.63 × 10^{-24}), apoplast (GO: 0048046) and photosynthesis organelles, indicating that most DEGs encode the extracellular and chloroplast localised proteins involved in stress adaptation. The enrichment of the apoplast suggests the activation of extracellular signalling and defence response under nitrogen deficiency.

The thylakoid-related terms, including GO: 0031977 (thylakoid lumen), GO: 0009543 (chloroplast thylakoid lumen), and GO: 0031978 (plastid thylakoid lumen), GO: 0009523 (photosystem II) and the oxygen-evolving complex (GO: 0009654), indicate chloroplast remodelling and modulation of the light dependent reaction. Nitrogen stress is known to alter the carbon metabolism and photosynthesis (Hakeem et al. 2012) and reduce the chlorophyll synthesis and thylakoid protein including RuBisCO (Masclaux-Daubresse et al. 2010). The suppressed expression of the photosynthesis related genes observed here is consistent with the findings in barley (Wei et al. 2016) and cotton (Iqbal et al. 2020), suggesting a conserved mechanism to decrease the energy expenditure in nitrogen deficiency. As the carbon and nitrogen metabolism are tightly coordinated (Foyer et al. 2001), the reduced photosynthetic activity likely reflects the adaptive metabolic adjustment. The enrichment of the apoplast and extracellular GO terms suggest further indicates that nitrogen sensing and stress signalling occurs outside the cell. The apoplast is a hotspot for stress perception and

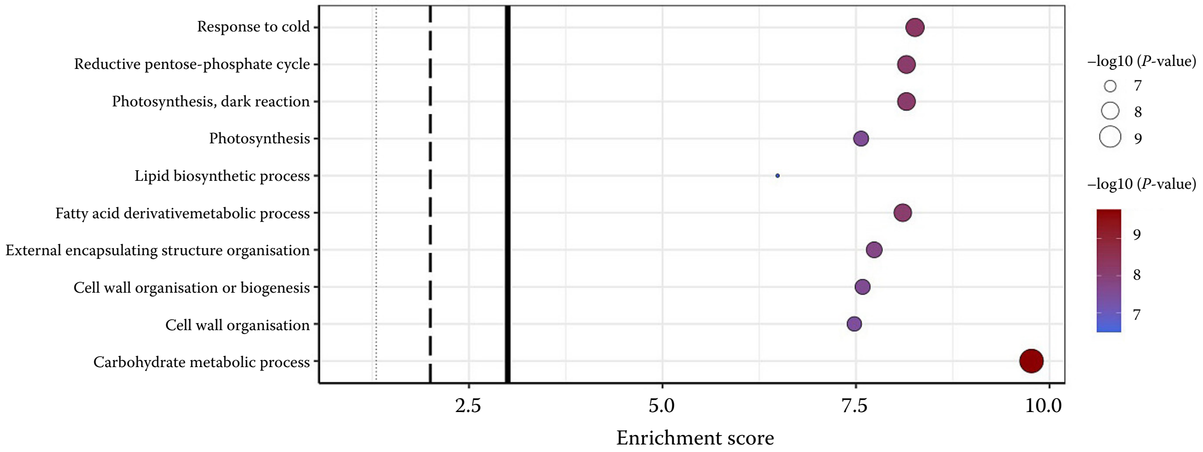

Figure 3. Gene ontology (GO) enrichment of the differentially expressed genes (DEGs) in the biological process (BP) category under nitrogen deficiency

The top ten significantly enriched GO terms (*P*-value) are shown; bubble size and colour represent –log10 (*P*-value), indicating the enrichment significance; vertical dashed lines denote the *P* -values thresholds (0.05, 0.01, and 0.001)

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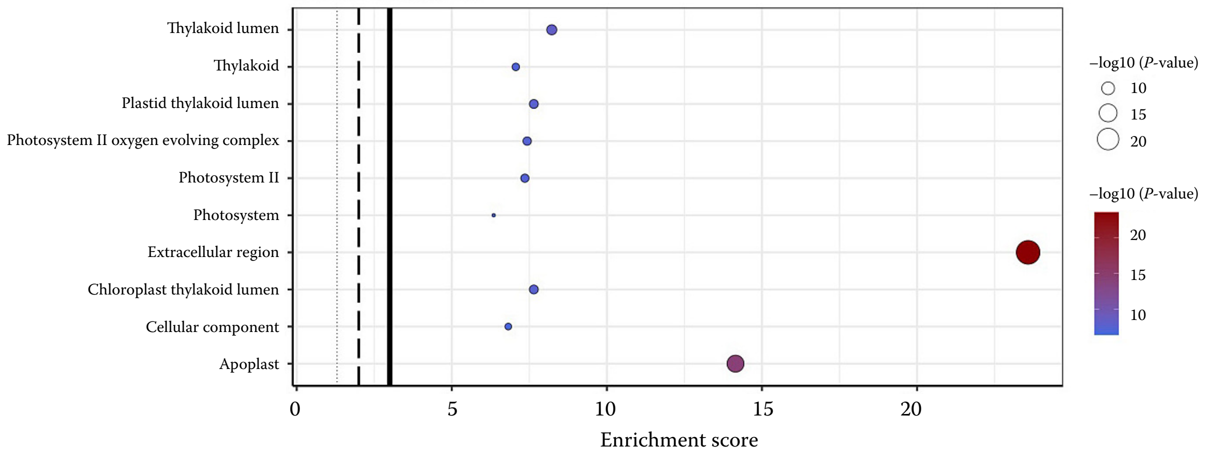

Figure 4. Gene ontology (GO) enrichment of the differentially expressed genes (DEGs) in the cellular component (CC) category under nitrogen deficiency

The top ten significantly enriched GO terms (*P*-value) are shown; bubble size and colour represent –log10 (*P*-value), indicating the enrichment significance; vertical dashed lines denote the *P*-values thresholds (0.05, 0.01, and 0.001)

ROS-mediated signalling. The enrichment of the oxidoreductase and peroxidase genes supports the activation of reactive oxygen species (ROS)-related pathways, consistent with reports of ROS accumulation under nitrogen deficiency (Chang et al. 2020; Zhao et al. 2021). Together, these results suggest that the shallot responds to nitrogen stress through the coordinated regulation of the extracellular signalling, chloroplast function and oxidative balance.

The GO enrichment analysis of the molecular function category identified 115 enriched terms, with the top ten shown in Figure 5; and further listed in Table S6 in the ESM. The most significant enriched term was the oxidoreductase activity (GO: 0016491; $P = 8.15 \times 10^{-9}$), indicating that many DEGs encode the redox related enzymes. This highlights the importance of oxidative stress response and ROS detoxification under nitrogen deficiency, as redox regulation is a central component of plant stress adaptation (Apel & Hirt 2004).

Other enriched functions included lactoperoxidase activity (GO: 0140825) and aldehyde decarbonylase

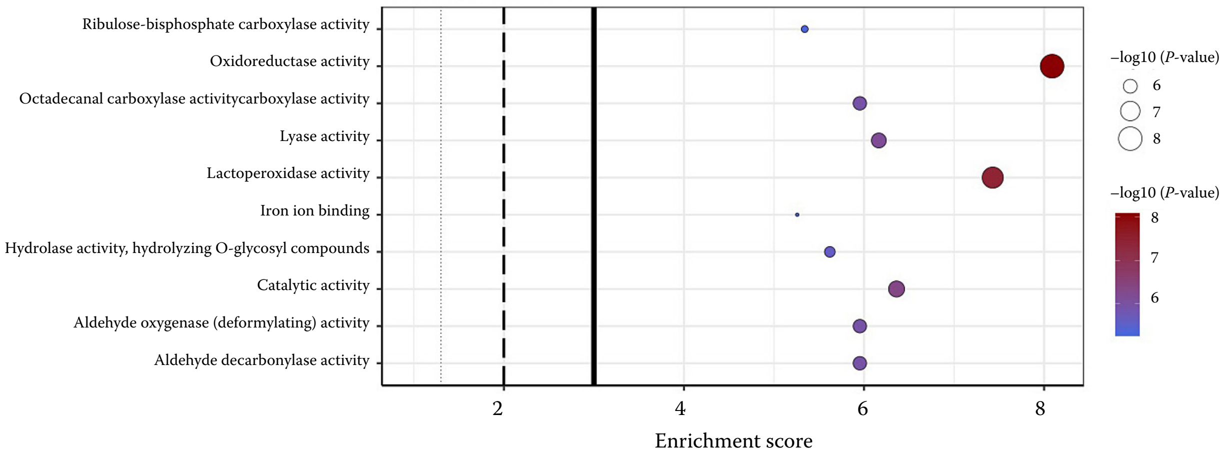

Figure 5. Gene ontology (GO) enrichment of the differentially expressed genes (DEGs) in the molecular function (MF) category under nitrogen deficiency

The top ten significantly enriched GO terms (*P*-value) are shown; bubble size and colour represent –log10 (*P*-value), indicating the enrichment significance; vertical dashed lines denote the *P*-values thresholds (0.05, 0.01, and 0.001)

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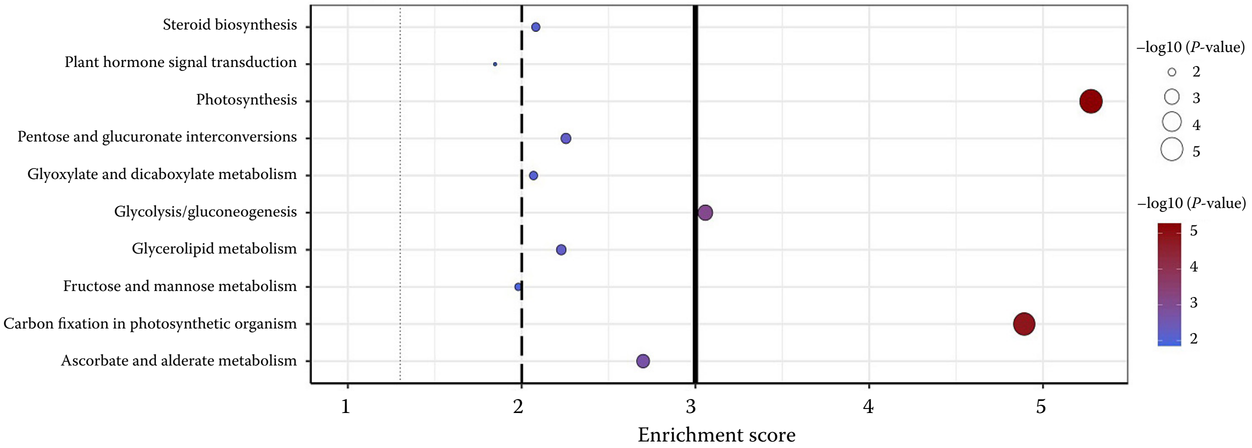

Figure 6. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways enrichment analysis of the differentially expressed genes (DEGs) under nitrogen deficiency

The top ten significantly enriched GO terms (*P*-value) are shown; bubble size and colour represent –log10 (*P*-value), indicating the enrichment significance; vertical dashed lines denote the *P*-values thresholds (0.05, 0.01, and 0.001)

related activities (GO: 0009924, GO: 0071771, and GO: 1990465), which are involved in oxidative metabolism, aldehyde detoxification metabolism and lipid derived signalling pathways often activated during stress. The enrichment of the ribulose-bisphosphate carboxylase activity (GO: 0016984) further indicates alterations in the carbon fixation and photosynthesis regulation. As the nitrogen availability strongly influences the photosynthetic enzyme and carbon nitrogen balance (Foyer et al. 2001), these results suggest that the coordinated metabolic reprogramming involves the redox control, enzymatic activity and photosynthesis under nitrogen stress.

The KEGG enrichment analysis (adjusted *P*-value < 0.05) identified many significant enriched pathways (Figure 6; Table S7 in the ESM). The photosynthesis was the most significant enriched pathway (gene Ratio 5/56; background 63/13850; adjusted *P*-value = 0.00058235), indicating strong modulation of the photosynthetic machinery under stress. The carbon fixation in photosynthetic organisms (ko00710) was also significantly enriched (gene ratio 4/56, adjusted *P*-value = 0.00070526), suggesting alternations in the Calvin cycle and photosynthetic adaptation.

Overall, the KEGG analysis identified photosynthesis and carbon fixation as the most affected pathways, confirming the reduced energy production under nitrogen deficiency. Nitrogen stress is known to impair photosynthetic efficiency and carbon fixation due to reduced synthesis of chlorophyll and key enzymes (Masclaux-Daubresse et al. 2010; Foyer et al. 2001), supporting our findings.

The enrichment of the translation, amino acid metabolism, carbohydrate metabolism, and the production of secondary metabolites indicates broader metabolic reprogramming. The activation of these pathways has been linked with stress tolerance via antioxidant defence and structural modification. These findings are supported by (Lu et al. 2021; Singh et al. 2022). Collectively, the KEGG results highlight that the shallot adapts to nitrogen stress by modulating the photosynthesis, primary metabolism and protective secondary metabolic pathways.

Gene set enrichment analysis. A gene set enrichment analysis (GSEA) was performed to identify the broader functional trends under nitrogen stress (Figure 7). Based on the normalised enrichment score (NES), two pathways were highlighted. The metabolic pathway (ko01100) comprised 40 genes and demonstrated moderate enrichment (ES = 0.290975; NES = 0.903846), indicating the general activation of the metabolic process. The leading-edge subset most comprised key metabolic genes such as *K00276*, *K01953*, *K00430*, *K18207*, *K21374*, *K00558*, and Table 2), suggesting coordinated adjustment of core biochemical pathways. Similarly, the biosynthesis of secondary metabolites (ko01110) exhibited enrichment (ES = 0.301832; NES = 0.860377), with 21 contributing genes (e.g., *K00276*, *K21374*, *K13496*, *K22013*, *K15404* and Table 2). Secondary metabolites pathways are commonly associated with stress adaptation and protective responses.

GSEA evaluated the gene expression at the pathway level by assessing whether predefined gene sets

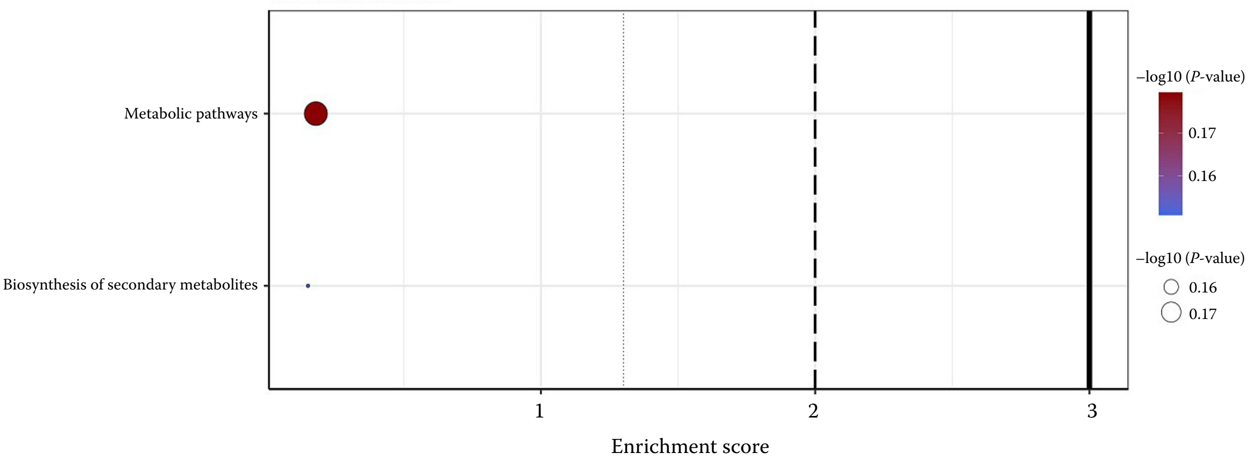

Figure 7. Kyoto Encyclopedia of Genes and Genomes (KEGG) gene set enrichment analysis of the differentially expressed genes (DEGs) under nitrogen deficiency

Bubble size and colour represent –log10 (*P*-value); vertical dashed lines denote the significance thresholds (0.05, 0.01, and 0.001)

are overrepresented at the top or bottom of a ranked gene list. If members of a gene set are found at the top (or bottom) of a ranked list, it indicates that the gene set is associated with the distinction. The ES reflect the cumulative contribution of the pathway genes across the ranked dataset (Reimand et al. 2019). Overall, these results suggest the coordinated modulation of the primary and secondary metabolism under nitrogen limitation.

qRT-PCR validation of differential gene expression. To validate the RNS-seq results, fifteen DEGs representing diverse functional categories were analysed by qRT-PCR. The expression patterns were highly consistent with the RNA sequence, confirming the reliability of the transcriptome profiling under nitrogen stress (Figure 8). Clear contrasting regulations were found between the normal and N-stressed plants.

Several genes showed significant induction in nitrogen deficiency, consistent with the RNA seq fold change. For example, *UCH-4* was strongly up-regulated in the stressed plants. These induced genes are generally associated with stress signalling, detoxification, and early defence activation, processes commonly intensified during nitrogen deficiency (Masclaux-Daubresse et al. 2010). This induction supports the enrichments of the stress-responsive pathway observed in this study.

Table 2. Gene set enrichment under nitrogen stress

ID	Description	ES	NGS	Adjusted *P*-value	Gene ID
ko01100	metabolic pathways	0.290975	0.903846	0.706599	K00276/K01953/K00430/K00430/K00276/K18207/K21374/K00558/K00430/K00002/K02639/K05298/K00855/K02717/K08901/K03542/K00847/K01051/K01915/K08679/K03146/K03841/K01792/K09828/K15920/K15397/K01792/K00454
ko01110	biosynthesis of secondary metabolites	0.301832	0.860377	0.706599	K00276/K01953/K00430/K00430/K00276/K21374/K00430/K00002/K13496/K00847/K22013/K03841/K01792/K09828/K15397/K01792/K00454/K15404

Gene set enrichment analysis (GSEA) was performed to identify the significantly enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways under nitrogen stress; the enrichment score (ES), number of genes in the gene set (NGS), adjusted *P*-value, and associated KEGG gene IDs are shown

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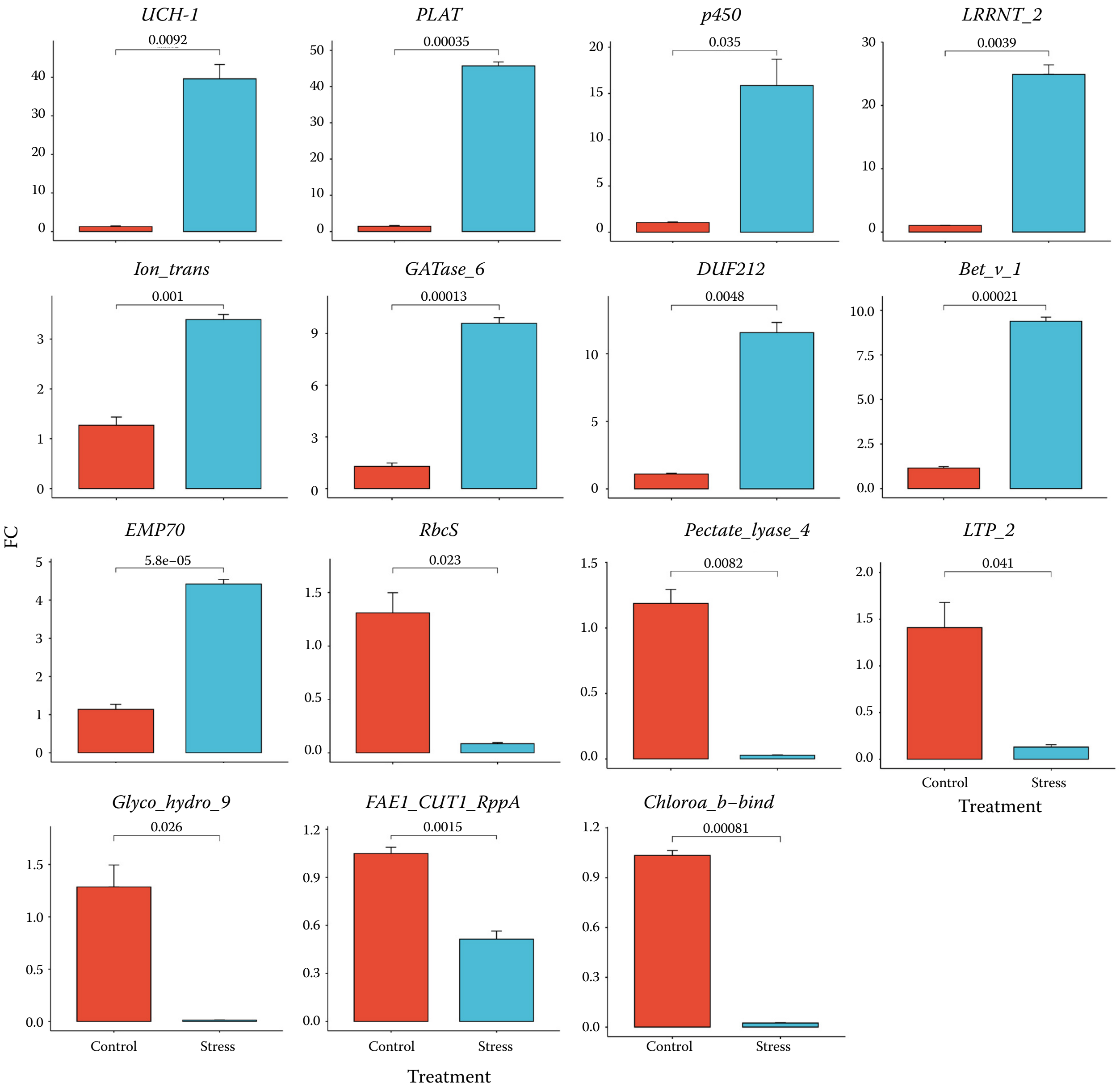

Figure 8. qRT-PCR validation of the selected differentially expressed genes (DEGs) in the shallots under nitrogen deficiency The relative expression levels are presented as the mean ± SD (*n* = 3 biological replicates); the expression levels of the genes were presented in fold change (FC); the bars represent the standard deviation (SD) values; Student's *t*-test was used to compare the gene expression levels, and the results are presented with the *P*-value above each bar

Conversely, many genes were markedly down-regulated under N stress. *EMP70* revealed strong repression deficient plants. Many suppressed genes were associated with the cell wall organisation, lipid transport, chlorophyll binding and photosynthetic metabolism. The pattern agrees with the previous reports showing that nitrogen stress suppressed the growth related and photosynthesis related genes to overcome energy expenditure (Zhang et al. 2020; Foyer et al. 2001).

The overall expression trends align with known nitrogen responsive regulatory networks. N deficiency activates key transcription factors likely NIN-like proteins (NLPs), basic leucine zipper (bZIP), myeloblastosis-related DNA-binding proteins (MYB) and WRKY-domain transcription factors (WRKY) families, which regulate nitrate sensing, assimilation and stress adaptation. In addition, calcium-mediated signalling and protein kinase also play key role in adjusting the leaf physiology under low nitrogen. Moreover, the differential expression of genes linked with photosystem II, thylakoid proteins and other chloroplast related factors suggested that the nitrogen deficiency perturbs the photochemical process and triggers the chloroplast to nucleus retrograde signalling. Together these transcription factors and signalling genes constituted a regulatory network and module metabolic adjustments, photosynthetic tuning, and stress adaptation under nitrogen deprivation. Incorporating this prospective strengthens the biological interpretation of the present findings.

The strong concordance between RNA seq and qRT-PCR confirms the robustness of the transcriptomic datasets. Similar high correlation between RNA seq and qRT-PCR has been widely reported (Shi & Gu 2020), supporting that the observed transcriptional changes represent biologically meaningful responses to nitrogen deprivation in shallot leaves.

As a whole, the transcriptomic outcomes clearly showed that the nitrogen limitation in shallots induces coordinated transcriptional reprogramming involving the suppression of photosynthesis-related genes (*Rbcs*) and the activation of stress responsive, redox regulating and nitrogen remobilisation pathways. The enrichment of the GO term related to the chloroplast function, oxidoreductase activity and extracellular signalling, along with the KEGG pathways linked with the photosynthesis and carbon fixation, highlight the tight coordination between the carbon and nitrogen metabolism under stress conditions. These changes suggest an adaptive mechanism in which the plant conserves energy by down-regulating the growth associated process while reducing the internal nitrogen recycling and stress defence. Furthermore, the strong agreement between the RNA-Seq and qRT-PCR validation supports the reliability of these findings. Collectively, these results provide a well-supported basis for concluding that the shallot adapts to nitrogen deficiency via integrated metabolic adjustments and stress responsive regulation.

CONCLUSION

This study provides the first transcriptomic insights into nitrogen deficiency in shallots, identifying 438 DEGs, that are predominantly down-regulated, reflecting the metabolic adjustment to the limited nitrogen availability. The GO, KEGG and GSEA analyses revealed the significant modulation of the pathways related to photosynthesis, amino acid metabolism extracellular signalling and oxidative stress. The enrichment of the chloroplast and apoplast related components, along with the oxidoreductase activity, highlights the coordinated regulation of the photosynthesis, redox balance and stress responses. The qRT-PCR validation confirmed the reliability of the RNA-seq data. These findings provide valuable molecular insights into nitrogen responsive networks in shallots and establish a foundation for future functional studies and breeding strategies to improve the nitrogen use efficiency.

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