Effects of nitrogen levels on gene expression and amino acid metabolism in Welsh onion

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Abstract

Background: Welsh onion constitutes an important crop due to its benefits in traditional medicine. Nitrogen is an important nutrient for plant growth and yield; however, little is known about its influence on the mechanisms of Welsh onion regulation genes. In this study, we introduced a gene expression and amino acid analysis of Welsh onion treated with different concentrations of nitrogen (N0, N1, and N2 at 0 kg/ha, 130 kg/ha, and 260 kg/ha, respectively).

Results: Approximately 1,665 genes were differentially regulated with different concentrations of nitrogen. Gene ontology enrichment analysis revealed that the genes involved in metabolic processes, protein biosynthesis, and transportation of amino acids were highly represented. KEGG analysis indicated that the pathways were related to amino acid metabolism, cysteine, beta-alanine, arginine, proline, and glutathione. Differential gene expression in response to varying nitrogen concentrations resulted in different amino acid content. A close relationship between gene expression and the content of amino acids was observed.

Conclusions: This work examined the effects of nitrogen on gene expression and amino acid synthesis and provides important evidence on the efficient use of nitrogen in Welsh onion.

Keywords: Welsh onion, Nitrogen, Transcriptome, Amino acids

Background

Nitrogen (N) is considered one of the most important nutrients required for plant growth and yield [1, 2]; therefore, crop yield and productivity have a strong relationship with the supply of nitrogen. The demand is fulfilled by the application of nitrogen fertilizers in the field, which come in various chemical forms, such as inorganic NO₃⁻, NH₄⁺, and organic urea, which is the most commonly applied nitrogen fertilizer worldwide [3]. However, excessive use of urea increases production costs and environmental pollution. Therefore, increasing nitrogen use efficiency (NUE) is important for sustainable agriculture.

Welsh onion (Allium fistulosum L.) is an important economical crop widely cultivated throughout the world, particularly in Asian countries [4]. It is often used as an ingredient for its special flavor and aroma and is considered a good source of nutrition; it is also used in traditional medicine [5]. However, despite its nutritional and medicinal value, information about this non-model plant's response to nitrogen is limited.

NUE in plants is highly complex and can induce diverse processes at both physiological and molecular levels. Ribonucleic acid-sequencing (RNA-seq) technology is a powerful...
tool that has been widely used to quantify gene expression levels in biological studies. RNA-seq was successfully applied in discovering key genes in populus [6], cucumber [7], Arabidopsis thaliana, and wheat [8, 9]. It was also used in genomics studies on A. fistulosum [5, 9], where the gene expression of different varieties of A. fistulosum was used [10]. To date, transcriptome studies have been carried out on many crops after nitrogen treatment, including Arabidopsis [11, 12], maize [13], poplar [14], and cucumber [7].

Amino acids are important nitrogen storage compounds in plants [15]. As the biosynthesis of amino acids requires nitrogen and carbon elements, nitrogen nutrition [16] and photosynthesis [17] are crucial, and after the uptake of nitrogen, glutamate synthase (GOGAT) and glutamine synthetase (GS) play vital roles in nitrogen assimilation in plants [18]. The relationship between specific genes and amino acids has been reported in other crops [19]. The abundance of nitrogen can strongly affect the biosynthesis of amino acids of tea plants, thus influence tea quality [20]. However, little information is available regarding the metabolism of nitrogen and amino acids and the gene regulation network in A. fistulosum.

In this study, we introduced differentially expressed gene (DEG) regulations and amino acids to investigate the relationship between nitrogen supply and metabolism in Welsh onion. We used RNA-seq technology and measured amino acids to explore the gene regulation network in A. fistulosum. There was a close relationship between gene expression and the content of amino acids; therefore, specific DEGs might improve the understanding of the influence of nitrogen in Welsh onion.

**Results**

**Sequencing and de novo assembly**

To study the global transcriptional response of A. fistulosum to various urea concentrations (N0, N1, and N2), we analyzed samples subjected to various nitrogen treatments using RNA-seq. De novo assembly was performed using a Trinity assembler, and the length distributions of the contigs, transcripts and unigenes are shown in Table 1. The next generation short-read sequences were assembled into 536,449 transcripts with an average length of 822.26 bp. The transcripts were subjected to cluster and assembly analyses. The longest transcript was taken as the sample unigene for data. A total of 247,703 unigenes with an average length of 634.16 bp were obtained. The N50 values of the transcripts and unigenes were 1,343 and 948 bp, respectively. The GC content of Welsh onion unigenes was 37.62%.

**Functional annotation and classification**

All assembled unigenes were subjected to BLASTx similarity analysis with an E-value of 10−5 against different NCBI databases, including NR, Gene Ontology (GO), the Kyoto Encyclopedia of Genes and Genomes (KEGG), evolutionary genealogy of genes: Non-supervised Orthologous Groups (eggNOG), and Swiss-Prot. As shown in Table 2, there were 59,689 unigenes (24.1%) matched in the NR database, 22,292 (9%) annotated in the GO database, 5,901 (2.38%) annotated in the KEGG database, 56,192 (22.69%) matched in the eggNOG database, and 47,305 (19.1%) matched in the Swiss-Prot database.

**Differentially expressed genes in response to nitrogen**

Table 3 compares plants without nitrogen (N0), 369/364 DEGs were up/down-regulated in the Welsh onion treated with half-levels of nitrogen (N1), 414/367 DEGs were up/down-regulated after full levels of nitrogen treatment (N2), and 387/275 DEGs were up/down-regulated in the pseudostems after full levels of nitrogen treatment with N1 as the control. As shown by the Venn diagram (Figs. 1), 224 DEGs overlapped in N1 vs. N0 and N2 vs. N0.

Seven genes were identified involving in N-uptake or assimilation from the transcriptome data. The lowest expression level of nitrate reductase (NR) and the highest expression level of GS corresponded to plants treated with the highest nitrogen concentration (Fig. 2). Down-regulation was observed for argininosuccinate synthase (AsuS) in all the groups. The order of the Welsh onion, according to the expression level of argininosuccinate lyase (ASL) and GOGAT, was N1>N2>N0, whereas the order for glutamate dehydrogenase (GDH) was the opposite (N1<N2<N0). The expressions of ammonium transporter (AMT) were not differential in any of the groups in response to nitrogen treatment.

**Table 1** Summary of sequence assembly for A. fistulosum

| Contig | Transcript | Unigene |
|--------|------------|---------|
| Total length (bp) | 215,015,755 | 441,101,605 | 157,084,336 |
| Sequence number | 703,130 | 536,449 | 247,703 |
| Max. length (bp) | 15,342 | 15,675 | 15,675 |
| Mean length (bp) | 305.80 | 822.26 | 634.16 |
| N50 (bp) | 424 | 1343 | 948 |
| N50 sequence no. | 107,314 | 94,254 | 41,994 |
| GC% | 37.72 | 37.47 | 37.62 |

**Table 2** Functional annotation of A. fistulosum transcriptome

| Database | Number | Percentage |
|----------|--------|------------|
| NR       | 59,689 | 24.10      |
| GO       | 22,292 | 9.00       |
| KEGG     | 5,901  | 2.38       |
| eggNOG   | 56,192 | 22.69      |
| Swiss-Prot| 47,305 | 19.10      |
| All databases | 3,260 | 1.32 |
The DEGs in comparative conditions were subjected to GO enrichment analysis to predict the biological functions of candidate genes in response to nitrogen. These transcripts were further classified into three major categories, but most of the assignments belonged to biological processes and molecular function. Significant GO terms were selected with a cut-off \( P \)-value of 0.05. Only one term (GO:0004766) belonging to molecular function was obtained between nitrogen-free and full levels of nitrogen treatment (data not shown). Six GO terms were classified as molecular function, and 12 GO terms were classified as biological processes (Tables 4 and 5) in the other two comparable groups. Among them, six terms were related to the biosynthesis of peptides or amino acids (GO:0004766, GO:0006412, GO:0043043, GO:0043604, GO:0006518, GO:0043603), while one term was related to the biosynthesis of nitrogen compound (GO:0044271). Four terms were related to transporter activity (GO:0080161, GO:0010329, GO:0015562, GO:0009926). These results indicated that candidate genes were closely related to the metabolism, biosynthesis, and transportation of nitrogen, and especially with amino acid metabolism.

KEGG pathway enrichment analysis was performed to categorize the biochemical pathways of DEGs. To better understand the biological functions and pathways of candidate genes in the Welsh onion with different N-supplements, all of the DEGs were annotated in the KEGG database. The pathways with \( P \)-value < 0.05 were regarded as significant. In terms of the enrichment analysis of the DEGs between nitrogen-free and half nitrogen treatment, two pathways (ko00190 and ko00904) were detected, which related to oxidative phosphorylation and diterpenoid biosynthesis, respectively (data not shown). Our results revealed that 21 pathways were involved after nitrogen treatment (Tables 6 and 7), with four similar pathways among different nitrogen concentrations (ko00270, ko00410, ko00330, ko00480). Interestingly, all of these pathways were related to amino acid metabolism. All the findings indicated that the level of nitrogen can affect the biosynthesis of amino acids.

### Regulation of amino acid content in response to different concentrations of nitrogen

After nitrogen treatment, the pseudostems contained significantly more threonine and proline than the nitrogen-free group (Fig. 3). The amounts of two amino acids were similar after different concentrations of nitrogen. The contents of glutamate, alanine, lysine, and histidine did not show marked differences with altered levels of nitrogen. Meanwhile, higher contents of cysteine and arginine were detected in the nitrogen-free samples. Higher contents of cysteine may attribute to the higher expression level of cysteine synthase (CS) corresponded to nitrogen-free group (data not shown). A higher expression level of genes was observed that encoded spermidine synthase involved in the biosynthesis pathway of proline, beta-alanine, cysteine, arginine, and glutathione (Tables 5 and 8). As the precursor of spermidine, decreased level of arginine was detected after nitrogen treatment. Alpha-enolase and glyceraldehyde-3-phosphate dehydrogenase genes were also up-regulated. To show the effects of the candidate genes, we proposed a regulation network of nitrogen metabolism and amino acid synthesis (Fig. 4).

### Discussion

Transcriptome analysis is an effective approach in identifying metabolic pathways and novel plant genes without a genomic sequence [21, 22]. The importance of using nitrogen efficiently in plants’ growth and yield has become a very attractive scientific topic. NUE includes the uptake and assimilation of nitrogen into plants, thus, identifying the genes involved in this process is currently

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**Table 3** Differentially expressed genes after nitrogen treatment

| Control   | Case   | Up-regulated genes | Down-regulated genes | Total DEGs |
|-----------|--------|--------------------|----------------------|------------|
| N0 vs N1  | N0     | 369                | 364                  | 733        |
| N0 vs N2  | N0     | 414                | 367                  | 781        |
| N1 vs N2  | N1     | 387                | 275                  | 662        |

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**Fig. 1** Venn diagram showing differentially expressed genes after nitrogen treatment: N0: nitrogen-free; N1: half nitrogen; N2: full nitrogen.
a key step. Several genes involved in the use of nitrogen were reported in *Arabidopsis* and rice [15], but although Welsh onion is an important crop, little is known about the role of these types of genes. In this study, RNA-seq was used to investigate the genes involved in NUE in response to different concentrations of nitrogen. Differential expression behavior of genes involved in N-uptake and assimilation and urea cycle were detected in response to nitrogen treatment (Fig. 4). Genes encoding for enzymes of N-assimilation, including NR and GDH, and gene for AsuS of urea cycle were found to be down-regulated. However, genes for GS and GOGAT of N-assimilation, and ASL of urea cycle were up-regulated after nitrogen supply.

**Table 4** GO enrichment analysis of the DEGs between nitrogen-free and half nitrogen (*P* < 0.05)

| Category             | GO_Term          | Annotation                                | Gene ratio | Background ratio | *P*-value |
|----------------------|------------------|-------------------------------------------|------------|------------------|-----------|
| molecular_function   | GO:0008161       | Auxin transmembrane transporter activity  | 2/733      | 4/22,292         | 0.00267   |
| molecular_function   | GO:0010329       | Auxin efflux transmembrane transporter activity | 2/733      | 4/22,292         | 0.00267   |
| molecular_function   | GO:0015562       | Efflux transmembrane transporter activity  | 2/733      | 5/22,292         | 0.00445   |
| biological_process   | GO:0010252       | Auxin homeostasis                         | 2/733      | 4/22,292         | 0.00658   |
| biological_process   | GO:0010817       | Regulation of hormone levels               | 3/733      | 38/22,292        | 0.02225   |
| biological_process   | GO:0009926       | Auxin polar transport                      | 2/733      | 10/22,292        | 0.04891   |
Nitrogen uptake includes the assimilation and remobilization of organic nitrogen in the whole plant [23]. Urea had a repressive effect on NO$_3^-$ influx but promoted NH$_4^+$ uptake in Arabidopsis [24]. This was consistent with the phenomena introduced in Welsh onion after urea treatment with a down-regulated expression pattern of a nitrogen uptake gene (NR) (Fig. 2). NR is a key enzyme that catalyzes the first step of nitrate assimilation, and its reduced expression under nitrogen stress indicated the regulation of this enzyme in rice [25]. The down-regulated pattern was also detected in nitrogen-deficient physic nut [26]. In this study, NR expression showed a reducing trend related to nitrogen concentration, showing a similar regulation pattern in A. fistulosum.

Several works on the correlation between gene expression and the biosynthesis of amino acids under nitrogen supplementation conditions have been conducted on Arabidopsis [27], rice [28], and tea [19]. In this study, we identified 1,665 DEGs in the pseudostems of Welsh onion in response to different concentrations of nitrogen. These DEGs provided candidate genes for further analysis of biological function and pathways regarding nitrogen transportation and metabolism. GO enrichment analysis efficiently predicted the biological functions according to the transcriptome data [29]. Multiple GO

| Category          | GO_Term               | Annotation                              | Gene ratio | Background ratio | P-value  |
|-------------------|-----------------------|-----------------------------------------|------------|-----------------|----------|
| molecular_function| GO:0004766            | Spermidine synthase activity            | 16/662     | 1,154/22,292    | 0.00848  |
| molecular_function| GO:0005198            | Structural molecule activity            | 15/662     | 1,027/22,292    | 0.000295 |
| molecular_function| GO:0003735            | Structural constituent of ribosome      | 20/662     | 2,310/22,292    | 0.000373 |
| biological_process| GO:0044271            | Cellular nitrogen compound biosynthetic process | 15/662     | 1,506/22,292    | 0.00241  |
| biological_process| GO:0006412            | Translation                             | 15/662     | 1,512/22,292    | 0.00683  |
| biological_process| GO:0043043            | Peptide biosynthetic process            | 2/662      | 6/22,292        | 0.00715  |
| biological_process| GO:0043604            | Amide biosynthetic process              | 15/662     | 1,555/22,292    | 0.00981  |
| biological_process| GO:0006518            | Peptide metabolic process               | 15/662     | 1,569/22,292    | 0.01084  |
| biological_process| GO:0010467            | Gene expression                         | 18/662     | 2,186/22,292    | 0.0133   |
| biological_process| GO:0043603            | Cellular amide metabolic process        | 15/662     | 1,654/22,292    | 0.01946  |
| biological_process| GO:0034645            | Cellular macromolecule biosynthetic process | 18/662    | 2,282/22,292    | 0.02289  |
| biological_process| GO:0009059            | Macromolecule biosynthetic process      | 18/662     | 2,313/22,292    | 0.02708  |

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terms were significantly represented under different levels of nitrogen treatment, especially those for amino acid and peptide biosynthesis, metabolism, and transporters. Moreover, KEGG analysis showed that a substantial number of DEGs were detected under nitrogen starvation and led to significant changes in some metabolic pathways, including amino acid metabolism, translation, carbohydrate metabolism, and biosynthesis of other secondary metabolites. Our previous results indicated that the highest phenolic content was observed in the N2 treatment group, whereas that in the N0 group was the lowest [30]. This can be attributed to the DEGs identified in the pathways of metabolism of terpenoids and polyketides between nitrogen-free and full levels of nitrogen treatment (Table 6).

The results revealed that the pseudostems of the Welsh onion, after nitrogen treatment, contained significantly more threonine and proline than the nitrogen-free group, while a higher content of cysteine and arginine were detected in the nitrogen-free samples. Cysteine and glutathione metabolism are reported to indicate parallels with Allium flavour precursor biosynthesis [31]. Moreover, cysteine is the first organic product generated from S [32]. Cysteine synthase is the last enzyme of sulfate assimilation pathway, and O-acetylserine (OAS), the precursor of cysteine, is derived from the carbon and nitrogen assimilation pathways [33]. The down-regulated pattern of CS was detected after nitrogen treatment. For one hand, the synthesis of cysteine decreased; for another, with the precursor of cysteine, flavour compounds were synthesized. The results indicated that nitrogen supply affect sulphur assimilation pathways, including the synthesis of cysteine and other products, thus affecting the flavour of Welsh onion. Ornithine is the point of entry for the biosynthesis of polyamines such as putrescine, spermidine, and spermine, which are used to store excess organic nitrogen in plant tissues [34]. Arginine is the precursor of ornithine, and this was probably the reason why the arginine content was reduced after nitrogen treatment (Fig. 4). The alpha-enolase and glyceraldehyde-3-phosphate dehydrogenase genes were also up-regulated. These are key enzyme genes related to glycolysis, and the catalytic products are the main precursors of amino acids. The number of precursors may determine the difference between the content of amino acids. It seems that nitrogen treatment might also promote glycolysis and tricarboxylic acid cycle (TCA) flux as well as amino acid metabolism. Our previous results showed that the highest yields were detected in the full levels of nitrogen

### Table 7
KEGG pathway enrichment analysis of the DEGs between half nitrogen and full levels of nitrogen ($P < 0.05$)

| Pathway ID | Pathway | Annotation | Gene ratio | Background ratio | P-value |
|------------|---------|------------|------------|------------------|---------|
| ko00270    | Cysteine and methionine metabolism | Amino acid metabolism | 3/662 | 22/5,901 | 0.0005864 |
| ko00410    | Beta-alanine metabolism | Metabolism of other amino acids | 2/662 | 32/5,901 | 0.0012478 |
| ko00330    | Arginine and proline metabolism | Amino acid metabolism | 2/662 | 42/5,901 | 0.0021466 |
| ko00480    | Glutathione metabolism | Metabolism of other amino acids | 2/662 | 55/5,901 | 0.0036590 |
| ko04913    | Ovarian steroidogenesis | Endocrine system | 1/662 | 3/5,901 | 0.0050761 |
| ko03010    | Ribosome | Translation | 3/662 | 267/5,901 | 0.0086727 |
| ko00140    | Steroid hormone biosynthesis | Lipid metabolism | 1/662 | 6/5,901 | 0.0101291 |
| ko00830    | Retinol metabolism | Metabolism of cofactors and vitamins | 1/662 | 7/5,901 | 0.0118082 |
| ko0380     | Tryptophan metabolism | Amino acid metabolism | 1/662 | 20/5,901 | 0.0334053 |
| ko0980     | Metabolism of xenobiotics by cytochrome P450 | Xenobiotics biodegradation and metabolism | 1/662 | 21/5,901 | 0.0350490 |
| ko04020    | Calcium signaling pathway | Signal transduction | 1/662 | 24/5,901 | 0.0399647 |
| ko04022    | cGMP-PKG signaling pathway | Signal transduction | 1/662 | 30/5,901 | 0.0497285 |

Fig. 3 Contents of amino acids (mg/g fresh weight) in pseudostems after nitrogen treatment; significance values were expressed as * $P < 0.05$, ** $P < 0.01$.
treatment (N2) followed by half-levels of nitrogen (N1) [35]. Therefore, it is possible that nitrogen triggers a number of molecular and physiological events that lead to the increase of plant biomass, especially for carbohydrate metabolism, amino acid metabolism.

In the glutamine–glutamate cycle, GOGAT, GS, and GDH were the key enzymes regulating the amount of each compound. Glutamate was always used as the nitrogen source in the biosynthesis of nitrogen compounds [19]. Under nitrogen starvation, the transcript levels of GS and GOGAT were up-regulated, whereas GDH was found to be significantly down-regulated (Fig. 2). We also measured the metabolites of the glutamine–glutamate cycle and found that the glutamate content did not significantly change after treatment with different concentrations of nitrogen. It can be assumed that the higher expression level of the two genes with nitrogen treatment was induced by urea-derived ammonium through a positive feedback mechanism via the GS–GOGAT cycle.

### Conclusions
This work examined the influence of nitrogen in the activation of nitrogen-related genes and amino acid metabolism in Welsh onion for the first time. The transcriptome analysis of Welsh onion on different concentrations of nitrogen treatment revealed that 1,665 genes were significantly regulated. GO analysis revealed that the DEGs were associated with diverse processes. The metabolism and transporters of various amino acids were highly represented, indicating the processes involved with using nitrogen. KEGG analysis provided the enrichment pathways related to amino acid metabolism. However, the DEGs’ response to nitrogen application resulted in different contents of amino acids. With the introduction of the effect of nitrogen in gene expression and amino acid synthesis, this work provides important evidence of NUE in Welsh onion.

### Methods
**Plant materials and RNA extraction**
The *A. fistulosum* species was obtained from the Shandong Academy of Agricultural Sciences. Planting was
performed as previously described [35], and seedlings were grown under field conditions in Zangqiu district, Jinan city, Shandong Province, China. The soil type was classified as cinnamon soil and had a nitrogen concentration of 80.67 mg kg−1 before the experiment. The plants were treated with three different concentrations of urea, namely, nitrogen-free (without urea), half levels of nitrogen (130 kg ha−1), and full levels of nitrogen (260 kg ha−1). The treatments (0 N, half N, full N) were named N0, N1, and N2. The seedlings were grown in 15 independent plots, with five plots for each nitrogen concentration. The fertilizer was used four times during the growth stages of Welsh onion, on June 25, August 14, August 27, and September 9, 2017, respectively. Welsh onion was collected on October 1, 2017, and further analyses were performed. Of the five field plot replications, two plot replications were randomly selected for analyses were performed. Of the five field plot replications, two plot replications were randomly selected for each group to make biological replications for RNA extraction. Randomly selected samples of pseudostems in each group to make biological replications for RNA extraction. Randomly selected samples of pseudostems in the same plot were pooled together, immediately frozen in liquid nitrogen, and stored at -80 °C. Total RNA was extracted from the pseudostems using Trizol reagent (Invitrogen Life Technologies) following the manufacturers’ instructions. RNA quality was determined using the Agilent 2100 Bioanalyzer (Santa Clara, CA, USA).

Transcriptome sequencing
For each treatment, three micrograms of RNA of the tissues with different concentrations of nitrogen were used to obtain the cDNA libraries. Sequencing libraries were generated using the TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA, USA). To select the preferred cDNA fragments that were 200 bp in length, the library fragments were purified using the AMPure XP system (Beckman Coulter, Beverly, CA, USA). DNA fragments with ligated adaptor molecules on both ends were selectively enriched with an Illumina PCR Primer Cocktail in a 15-cycle polymerase chain reaction (PCR) reaction. The 150 bp paired-end cDNA libraries were sequenced on Illumina’s Hiseq 2500 (Shanghai Personal Biotechnology Cp. Ltd.) following standard Illumina methods.

Functional annotation
To obtain high-quality reads, the raw reads were filtered to remove low-quality reads and reads with adapters. The reads were assembled using Trinity software with default parameters [36, 37]. First, contigs were obtained by extension based on the overlap between sequences. Next, the contigs were joined into transcripts by paired-end mapping. Finally, the contigs were connected to get sequences that could not be extended at either end. The longest transcripts of each gene in the upper genome were extracted as the reference transcript sequence, and cuffcomapare software was used to compare the variable splice sequence of this project with the reference transcript sequence (gff), using ASTALAVISTA software to analyze the variable splicing event. Such sequences were defined as unigenes, which were then aligned with the NR, GO, KEGG, eggNOG, and Swiss-Prot databases.

Differential gene expression and gene enrichment analysis
To analyze differential gene expression, candidate genes were identified with DESeq software [38, 39] in each comparison. Transcripts that exhibited two-fold or above were considered as differentially expressed, and the P-value threshold was set to 0.05. Enrichment patterns were clustered by pheatmap software using a complete linkage method. The unigenes were aligned to the GO and KEGG databases to predict the possible functions and metabolic pathways involved. GO terms were assigned by the Blast2GO program [40]. The KEGG Automatic Annotation Server (KAAS) was used for pathway annotation. The database searches were performed using BLASTX [41] with a cut-off E-value of 10−5. The enrichment of terms in the different treatments was further analyzed (P < 0.05).

Amino acids detection
The samples for the analysis of amino acids were the same as those used in transcriptome sequencing. The pseudostem samples (0.5 g) with three biological replicates were treated with 5 mL of 10% acetic acid and grinding on ice. The extractions were centrifuged at 12,000 rpm for 20 min. The filtered liquid was collected to enable amino acid detection. All of the extracted filtrates were filtered through 0.45 μm membranes before being measured. Amino acids were quantified using an amino acid analyzer (Hitachi, L-8900, Japan) with standard methods. Statistical analyses were performed using OriginPro to evaluate the statistical significance of differences between different culture conditions. Analysis was conducted with three technical replicates, and error bars (± SEM) were shown for independent experiments. Significances were calculated using an ANOVA approach, and the Tukey test was applied to determine differences between treated and untreated samples. P-value < 0.05 was considered significant.

Abbreviations
N: Nitrogen; NUE: nitrogen use efficiency; RNA-seq: Ribonucleic acid sequencing; GOGAT: glutamate synthase; GS: glutamine synthetase; DEG: differentially expressed gene; GO: Gene Ontology; KEGG: the Kyoto Encyclopedia of Genes and Genomes; eggNOG: evolutionary genealogy of genes; Non-supervised Orthologous Groups; NR: nitrate reductase; ASuS: argininosuccinate synthase; ASL: argininosuccinate lyase; GDH: glutamate dehydrogenase; AMT: ammonium transporter; TCA: tricarboxylic acid cycle; PCR: polymerase chain reaction; KAAS: KEGG Automatic Annotation Server
Acknowledgements
We thank Prof. Rongzong Cui, Shandong Academy of Agricultural Sciences, for providing the plant materials.

Authors’ contributions
CZ conducted experiments, analyzed data, and wrote the manuscript. GM conducted experiments and analyzed data. LZ prepared samples and assayed the contents of amino acids. SZ, LS, and X5 analyzed data. OB-H revis ed the manuscript. KL supported the study. QY and LZ conceived and designed the research and wrote the manuscript. All authors read and approved the submitted version.

Funding
This work was financially supported by the Key Technologies Research and Development Program of China (No. 2016FY0200100), Key Technologies Research and Development Program of Shandong Province (No. 2019QYTPY024, 2018YYS022, 2019YYS019, 2018YYS007, 2018YFJH0404, and 2018XGC0204), and the Science, Education, and Industry Integration Innovation project at Qilu University of Technology (Shandong Academy of Sciences) (No. 2020KJC-GH10).

Availability of data and materials
RNA-seq raw data were deposited in the SRA database of NCBI with accession number PRJNA504406.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Received: 18 April 2021 Accepted: 28 October 2021
Published online: 07 November 2021

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