Journal of Plant Physiology 246–247 (2020) 153137

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

Transcriptome analysis of rice (*Oryza sativa* L.) in response to ammonium resupply reveals the involvement of phytohormone signaling and the transcription factor OsJAZ9 in reprogramming of nitrogen uptake and metabolism

Li Sun[a,b,1], Dong-Wei Di[a,1], Guangjie Li[a], Yilin Li[a], Herbert J. Kronzucker[c,d], Weiming Shi[a,*]

[a] State Key Laboratory of Soil and Sustainable Agriculture, Institute of Soil Science, Chinese Academy of Sciences, No.71 East Beijing Road, Nanjing, Jiangsu, 210008, China
[b] State Key Lab of Crop Genetics and Germplasm Enhancement, Cytogenetics Institute, Nanjing Agricultural University/JCIC-MCP, Nanjing, Jiangsu, 210095, China
[c] School of Agriculture and Food, The University of Melbourne, Parkville, VIC 3010, Australia
[d] Faculty of Land and Food Systems, University of British Columbia, Vancouver, BC, V6T 1Z4, Canada

ARTICLE INFO

Keywords:
Low ammonium (NH_4^+)
Rice (*Oryza sativa* L.)
RNA-Seq (quantification) profiling
Spatial-temporal codes
OsJAZ9

ABSTRACT

NH_4^+ is not only the primary nitrogen for rice, a well-known NH_4^+ specialist, but is also the chief limiting factor for its production. Limiting NH_4^+ triggers a series of physiological and biochemical responses that help rice optimise its nitrogen acquisition. However, the dynamic nature and spatial distribution of the adjustments at the whole plant level during this response are still unknown. Here, nitrogen-starved rice seedlings were treated with 0.1 mM $(NH_4)_2SO_4$ for 4 or 12 h, and then the shoots and roots were harvested for RNA-Seq analysis. We identified 138 and 815 differentially expressed genes (DEGs) in shoots, and 597 and 1074 in roots following 4 and 12 h treatment, respectively. Up-regulated DEGs mainly participated in phenylpropanoid, sugar, and amino acid metabolism, which was confirmed by chemical content analysis. The transcription factor *OsJAZ9* was the most pronouncedly induced component under low NH_4^+ in roots, and a significant increase in root growth, NH_4^+ absorption, amino acid, and sugar metabolism in response to resupplied NH_4^+ following nitrogen starvation was identified in *JAZ9ox* (OsJAZ9-overexpressed) and *coi1* (OsCOI1-RNAi). Our data provide comprehensive insight into the whole-plant transcriptomic response in terms of metabolic processes and signaling transduction to a low-NH_4^+ signal, and identify the transcription factor OsJAZ9 and its involvement in the regulation of carbon/nitrogen metabolism as central to the response to low NH_4^+.

1. Introduction

Most plants possess a fundamental dependence on inorganic nitrogen (N), and healthy plant growth depends upon a sufficient supply of N (Takehisa et al., 2013). When supplies are limited, normal development is restricted and crop biomass and yields are reduced (Good et al., 2004). In soil, nitrate (NO_3^-) and ammonium (NH_4^+) are the main forms of N available for plants. Nevertheless, crops use only a small quantity of the N that is applied during a given season (Socolow, 1999), and unused portions then leak into water reservoirs or are released into the atmosphere, producing significant environmental contamination (Gruber and Galloway, 2008; Min et al., 2012; Shi et al., 2010). Compared with NO_3^-, NH_4^+ is more easily absorbed by roots (Kronzucker et al., 2000). Due to the detrimental effects of adding high NO_3^- concentrations to ecosystems, the potential of NH_4^+ as an N source for agriculture has received increased attention in recent years, and the quest to improve N-use efficiency (NUE) while mitigating environmental impacts has been intensified (Sun et al., 2017; Swarbreck et al., 2019).

Rice (*Oryza sativa* L.), the major cereal crop in the world, is well-known as an NH_4^+ specialist (Kronzucker et al., 1999, 2000). NH_4^+ is not only the primary nutrient for rice growth and development in

Abbreviations: GO, gene ontology; KEGG, kyoto encyclopedia of genes and genomes; N, nitrogen; NH_4^+, ammonium; NO_3^-, nitrate; NUE, nitrogen use efficiency

* Corresponding author.
E-mail addresses: sunli@njau.edu.cn (L. Sun), dwdi@issas.ac.cn (D.-W. Di), gjli@issas.ac.cn (G. Li), ylli@issas.ac.cn (Y. Li), herbert.kronzucker@unimelb.edu.au, herbert.kronzucker@ubc.ca (H.J. Kronzucker), wmshi@issas.ac.cn (W. Shi).

[1] These authors contribute equally to this work.

https://doi.org/10.1016/j.jplph.2020.153137
Received 14 December 2019; Received in revised form 7 February 2020; Accepted 10 February 2020
Available online 22 February 2020
0176-1617/

paddies, but is also the chief limiting factor for its production (Yang et al., 2015). For rice systems, it has been estimated that less than 30–40 % of applied N fertilizer is absorbed by the crop, while most is lost through the processes of denitrification, volatilization, leaching, and runoff, which, in turns, causes pollution to the atmosphere and aquatic environments (Kronzucker et al., 2000; Min et al., 2012). Thus, improvements on N-nutrient management practices and breeding of rice varieties with high NUE or low N requirements are crucial for sustainable agriculture (Swarbreck et al., 2019). Limiting NH_4^+ can trigger an array of acclimation responses at the morphological, physiological, and biochemical levels, to help the plant increase N acquisition. With the emergence of omic technologies, some analyses of limiting NH_4^+ conditions (when NH_4^+ is provided as the sole N source and at low levels) at the genome-wide level have been conducted in rice. For instance, Chandran et al. (2016) reported that the TCA cycle and mitochondrial electron transport were affected in a tightly coordinated fashion when rice roots were subjected to low NH_4^+, and Yang et al. (2015) reported a coordinated regulation of carbohydrate and amino acid metabolism in the root and shoot after 4 h under N-free (no NH_4^+) conditions. Meanwhile, some genes associated with low-NH_4^+ environments have been identified. For example, *OsIDD10* regulates circuits of the metabolic and cellular response to low NH_4^+ by activating the transcription of *AMT1.2* and *GDH2* (Xuan et al., 2013). *OsAS1* can induce the biosynthesis of asparagine in rice roots under low NH_4^+ (Ohashi et al., 2015). *OsAMT1.1* transgenic rice with enhanced NH_4^+ permeability showed superior growth and higher yield under low-NH_4^+ conditions (Ranathunge et al., 2014). Recently, it was shown that *OsAMT1.1* significantly contributes to NH_4^+ uptake under low NH_4^+, offering the potential of improving NUE and grain yield in rice (Ranathunge et al., 2014). Similarly, *OsAMT1.3* has been shown to modify rice growth and carbon-nitrogen metabolic status under low-NH_4^+ conditions (Bao et al., 2015). These studies have significantly enhanced our understanding of the regulatory mechanisms underlying acclimation and adaptation to limiting NH_4^+, and provide an important molecular framework for improvements in NUE.

Plant fitness and survival largely depend on the ability to stage plastic responses to a changing environment through extended and connected signaling networks (Goossens et al., 2016). While hormones are major players in the establishment and interconnectedness of plant signaling networks (Verma et al., 2016). However, under limiting NH_4^+, not all phytohormones have been examined. For example, auxin (IAA) and strigolactones are important mediators in shoot branching suppression under low-N conditions in *Arabidopsis* (de Jong et al., 2014). Auxin and ethylene response pathways are related to the genotypic difference in low-N tolerance in barley (Quan et al., 2016). More generally, the auxin and cytokinin signaling pathways are centrally involved in regulating root elongation (Khan et al., 2015). The action of ethylene on N uptake and root growth is independent of other phytohormones, as low nitrate levels also increase cytokinins, auxin, and ABA (Khan et al., 2015). Jasmonic acid (JA) accomplishes unique roles in plant developmental processes including flower development, embryogenesis, seed germination, fruit ripening, leaf senescence and responses to environmental stress, such as low temperature, salinity, drought, wounding, heavy metal toxicity, and attack by pathogens and insects (Sharma and Laxmi, 2016). However, its function in improving NUE under limiting NH_4^+ is not known.

To date, studies on the response to low NH_4^+ have been limited to specific tissues or specific metabolic changes, failing to capture whole-plant responses and dynamic and spatial adjustments. Moreover, signaling transduction changes between root and shoot, and associated feedback mechanisms, have not been investigated. A whole-genome analysis of the spatio-temporal responses to limiting NH_4^+ conditions, at more than one time point, was conducted to address this deficiency, and provide insight into the global response of hormonal regulation networks under this important nutritional condition. Most previous studies considered shifts from normal N to limiting N, focusing on low-N stress, or shifts from N starvation to re-supply for the examination of NUE (Chandran et al., 2016; Yang et al., 2015). To better understand how rice tissues respond to NH_4^+ re-supply at low levels of provision, we utilized Illumina HiSeq™ 2000 RNA sequence (RNA-seq) and characterized shoot and root transcriptomes following shifts from N-starved conditions to re-supply of low NH_4^+; NH_4^+ under such low levels of provision in a resupply design can act to magnify signaling processes in N utilization. We have addressed the following two key research questions: (1) Which metabolic processes and signaling pathways are involved in the response to low-NH_4^+ re-supply in rice? (2) Which key genes participate in regulating rice growth under low-NH_4^+ conditions?

2. Materials and methods

2.1. Plant growth conditions and low-NH_4^+ treatment

Seeds of *Oryza sativa* L. were surface-sterilized with 3% H_2O_2 for 30 min, washed extensively with distilled water, and then germinated in distilled water at 28 °C for 2 days. Germinated seeds were transferred into modified Johnson's solution (2 mM $MgSO_4$; 1 mM $CaCl_2$; 0.5 mM KCl; 0.3 mM NaH_2PO_4; 0.1 mM Fe-EDTA; 20 μM H_3BO_3; 9 μM $MnCl_2$; 1.5 μM $CuSO_4$; 1.5 μM $ZnSO_4$; 0.5 μM Na_2MoO_4,1 mM $(NH_4)_2SO_4$, pH 5.5) for another 14 days. Seedlings were grown in a phytotron with 16 h/8 h (28/25 °C) day/night and a light intensity of 400 micro mol m^{-2} s^{-1}, as described by Sun et al. (2017). Then, 14-day-old seedlings were transferred to 0 N solution for another 3 days to deplete endogenous N. After this, seedlings were transferred to a solution containing 0.1 mM $(NH_4)_2SO_4$ for further growth. The root length, fresh weights of shoots and roots were determined. The shoot and root were collected separately at 0, 4, and 12 h after re-supply of a 0.1 mM $(NH_4)_2SO_4$ treatment and were used to RNA-Seq (Quantification) analysis.

2.2. Tissue NH_4^+ determination

Roots (approximately 1 g) were collected and desorbed for 5 min in 10 mM $CaSO_4$, to remove extracellular free NH_4^+, then were homogenized under liquid nitrogen by a mortar and pestle, with the addition of 6 mL of 10 mM formic acid. Contents of free NH_4^+ were determined using a modified OPA (o-phthalaldehyde) method, as described elsewhere (Sun et al., 2017).

2.3. Sugar, amino acid, and flavonoid content determination

For sugar analysis, 1 g fresh weight tissue was frozen in liquid nitrogen immediately after each treatment, then dissolved with 50 mL of distilled water, followed by slow addition of 5 mL of zinc acetate and 5 mL of potassium ferricyanide solution. Then, distilled water was added to produce a total volume of solution of 100 mL, and the solution was stirred by magnetic force for 30 min. After placing the solution at room temperature, it was filtered with dry filter paper, then about 2 mL filtrate was collected with a 0.45 μm microporous filter membrane for liquid filtration and chromatography (Wang et al., 2002).

For amino acid analysis, 0.6 *g* (fresh weight) tissue was frozen in liquid nitrogen immediately after each treatment. Then, 10 mL 50 % ethanol solution (containing 0.01 mmol hydrochloric acid) was added and subjected to a water bath and ultrasound at 4 °C for 30 min. Then, centrifugation occurred at 12,000 rpm at 4 °C for 5 min, and about 1 mL of extract was filtered with a 0.22 μm filter membrane and then used in a SYKAM Amino Acid Analyzer for further analysis (Sykam, Germany) (del Campo et al., 2009).

For flavonoid analysis, extracts were screened for flavonoids by a protocol described elsewhere (Ordonez et al., 2006). Briefly, 1 mL of ammonia solution was added to 0.2 mL of extract and mixed. Then, 1 mL of concentrated H_2SO_4 was added to this mixture and the development of yellow color was recorded.

2.4. Isotope analysis

^{15}N-labeling concentration was determined as described in (Sun et al., 2016). Following imposition of a 3-day N-starvation protocol, 2-week-old seedlings were transferred to nutrient solution to which 0.1 mM $^{15}(NH_4)_2SO_4$ was added. After 4 h of treatment, shoots and roots were washed, separated, freeze-dried, ground into a powder, and subjected to a Thermo Flash 2000 analyzer hyphenated to a Thermo Fisher (Waltham, MA, USA) Delta-V isotope ratio mass spectrometer to determine ^{15}N abundance. Nitrogen uptake during the labeling was calculated by excess atom% ^{15}N in the plant tissue and N_{dff}% ^{15}N = excess atom% ^{15}N in the plant tissue/excess atom % of the tracer × 100 %.

2.5. Root characteristics

Root scanning was performed prior to the sampling process using an Epson Expression 10000XL 1.0 system (Regent Instruments Company, Canada) as reported in (Di et al., 2018). The parameters were recorded with a root image analysis system using the WinRHIZO software: total root length (mm), surface area (cm^2), total volume (cm^3).

2.6. RNA extraction and sequencing

Total RNA was extracted with TRIzol reagent (Invitrogen, USA) according to the protocol provided by the manufacturer. The methods for synthesizing and purifying first-strand cDNA and double-strands cDNA, and sample library construction were as described in detail in a previous study (Sun et al., 2017). The library products were further sequenced by the Illumina HiSeq™ 2000.

2.7. Mapping of RNA-Seq reads

The sequencing data ("raw reads"), which were produced from Illumina HiSeq™ 2000 RNA sequencing, were subjected to QC(quality control) to judge whether a re-sequencing step was neccesary. Then, raw reads were further filtered into "clean reads", and were stored in FASTQ format (Cock et al., 2010). The gene expression (FPKM) levels were calculated as described in a previous study (Sun et al., 2017).

2.8. Screening differentially expressed genes (DEGs) using NOISeq

DEGs were screened by the NOISeq method. The detailed steps were described in a previous study (Sun et al., 2017). DEGs were screened according to the default criteria, which was |log2 (fold change)| ≥ 2-fold and diverge probability ≥ 0.8.

2.9. Gene ontology (GO) functional annotation

The Web Gene Ontology Annotation Plot (WEGO) was used to perform the GO classifications. We mapped all DEGs to GO terms in the web resource http://www.geneontology.org/, and calculated the gene numbers for every term. DEGs were further assigned to "biological functions", "cellular component", and "molecular function" categories. The detailed method was described in Sun et al. (2017).

2.10. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis

The KEGG database (http://www.genome.jp/kegg/) was used to acquire enzyme commission (EC) numbers of DEGs. Then, we mapped EC numbers to the KEGG biochemical pathways and obtained KEGG Pathway-Maps (Sun et al., 2017).

3. The regulatory network analysis for low-NH_4^+-induced transcription factors (TFs) in roots

The regulated TFs in roots (4R: 4 -h-Root; 12R: 12 -h-Root) were chosen for the analysis if the regulatory network of TFs (q value < 0.05, score > 20). The TFs were first classified into protein families, and the binding sites were obtained from published studies. Then, the binding sites were blasted from 1500-bp upstream of all regulated genes, and the genes containing more than one binding site were selected and connected to the TFs with lines. Circle size indicates the log2Ratio.

3.1. Quantitative real-time PCR

Total RNA was extracted from shoots and roots harvested at the specified time points with TRIzol reagent (Invitrogen, USA). Total RNA (1 μg) was used for reverse transcription by M-MLV Reverse Transcriptase (Promega), and the cDNA samples were diluted 20-fold for further experiments. For qRT-PCR, a 20-μL reaction mixture (10 μL SYBR Premix Ex Taq (TaKaRa), 1.6 μL primers (0.8 μL/0.8 μL 10 μM forward/reverse primer), 1.6 μL cDNA template and 6.4 μL ddH_2O were amplified in a Light Cycler® 480II (Roche). The reaction protocol were described as follows: initial denaturation at 95 °C for 30 s, 40 cycles of amplification at 95 °C/60 °C for 10 s and 30 s, respectively. The melting curve was carried out in the 65 − 95 °C range. Each reaction was performed 3 times, and the means of data were normalized relative to UBI1. Primer sequences were as listed in Table S1.

3.2. Statistical analysis

All statistical analyses were performed using SPSS version 13.0, and one-way ANOVA was performed with a homogeneity of variance test, followed by an LSD test to check for quantitative differences between treatments. $P < 0.05$ was set as the significance cut-off. All data were analyzed by Prism 6 software. One-way ANOVA was performed among multiple groups, and $P < 0.05$ was considered as the significance cut-off. Student's *t*-test was used to analyze the significance between two groups. *P* values refer to different significances at: *P < 0.05, **P < 0.01and ***P < 0.001, respectively.

4. Results

4.1. Phenotypic response of N-starved rice to low NH_4^+

To observe the effect of low-NH_4^+ resupply on N-starved rice, seedlings N-starved for 3 days (17-day-old) were transferred to N-free medium with or without 0.2 mM ammonium for another 2 days and then analyzed the root growth. Our data show that seedlings continued to grow under both treatments, and that root length, root area, and root volume were increased 34.2 %, 73.2 %, and 123.9 % under 0.1 mM $(NH_4)_2SO_4$ conditions, compared with those in the 0 mM $(NH_4)_2SO_4$ condition (Fig. 1 a–d). We measured endogenous free NH_4^+ content in the roots and found that NH_4^+ levels were increased significantly at 4 h and increased` up to 2.1-fold at 12 h (Fig. 1e). Then, we analyzed the transcription levels of the genes encoding high-affinity NH_4^+ transporters from the *OsAMT1* gene family by qRT-PCR. The expression levels of *OsAMT1.1*, *OsAMT1.2,* and *OsAMT1.3* in the roots showed clear induction and reached their highest levels at 4 h. *OsAMT1.1* and *OsAMT1.2* maintained their high expression levels up to 12 h, while *OsAMT1.3* held high levels up to 48 h (Fig. 1f). Hence, we selected 0, 4, and 12 h for RNA-Seq analysis.

4.2. Identification of DEGs in response to low NH_4^+ resupply following N starvation

18 samples were sequenced by RNA-Seq technology, and the average number of raw sequencing reads and clean reads are shown

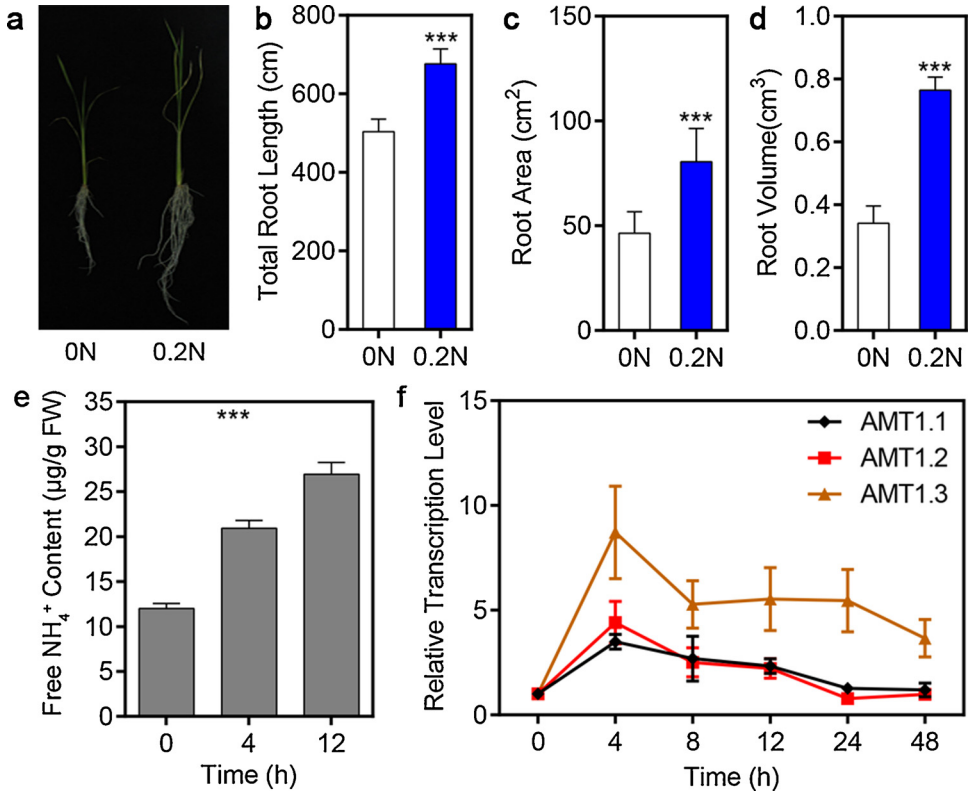


Fig. 1. Phenotypic response of N-starved rice to low NH_4^+ supplementation. The root phenotype **(a),** total root length **(b)**, root surface area **(c),** and root volume **(d)** following N starvation (0 N) and with 0.2 mM NH_4^+ supplementation (0.2 N) after N starvation (n = 15). NH_4^+ concentration **(e),** and expression levels of the NH_4^+ transporter genes *OsAMT1.1*, *OsAMT1.2,* and *OsAMT1.3* **(f)** in the roots with 0.2 mM NH_4^+ supplementation after N starvation (n = 3). Values are means ± SD. *** indicate significant difference at $P < 0.001$.

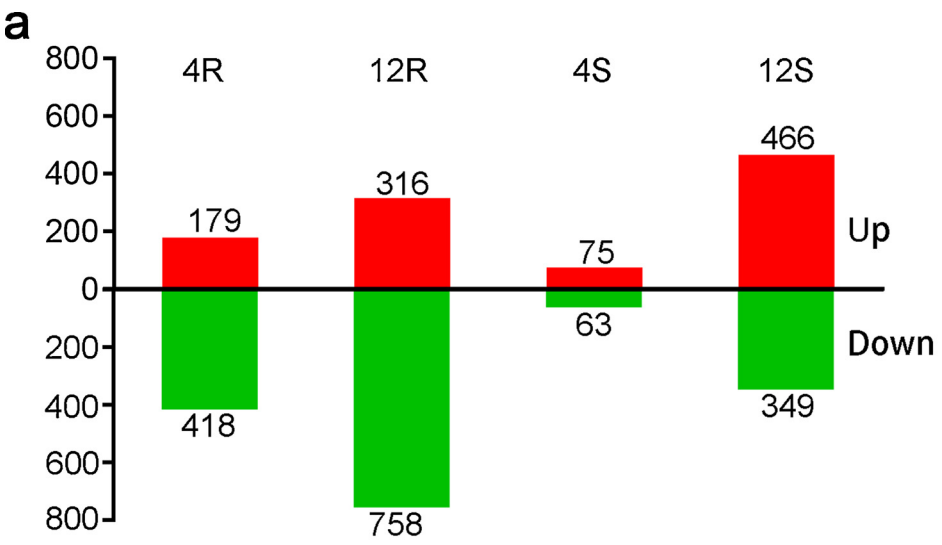
(Table S2). The unique mapping ratio with the reference gene and the average genome mapping ratio were more than 80 % (Table S2). To judge the differences in gene expression and to define DEGs, two filtering criteria were deployed in our data analysis: the fold change > 2 between any two treatments, and a *p-value* < 0.05. Four profiles (4S:4 -h-Shoot/12S:12 -h-Shoot and 4R:4 -h-Root/12R:12-h-Root) were utilized to depict the transcripts of all of the DEGs of the three replicates at 4 h and 12 h in both shoots and roots. Moreover, the qRT-PCR results of selected genes were in close accordance with those obtained by RNA-seq (Fig. S1).

In all, 75 and 466 DEGs were up-regulated, and 63 and 349 DEGs were down-regulated, based on the analysis of 4S and 12S, respectively (Fig. 2a). 179 and 418 DEGs were up-regulated and down-regulated, respectively, for 4R compared with 316 and 758 DEGs for 12R (Fig. 2a). To confirm both unique and common genes at the time points in both shoot and roots, numbers were calculated and presented using a Venn diagram (Fig. 2b-c). The results showed that 32 and 62 DEGs (> 2-fold) were commonly induced in the shoots and the roots at different time points, respectively. 3 DEGs (> 2-fold) were up-regulated in 4S, 4R, 12S and 12R, demonstrating a progressive biological process (Fig. 2b-c). Moreover, 28 and 651 DEGs were unique to the shoots, compared to 34 and 233 DEGs that were uniquely induced in the roots at 4 h and 12 h, respectively (Fig. 2c).

To investigate the functions of these DEGs, we mapped them in the GO database, and searched for obviously enriched GO terms (corrected *p-value* < 0.05) (Fig. S2). According to the "biological processes" classification, DEGs were mainly mapped to "metabolic process", "cellular process", "establishment of localization", "localization", "single organism process", and "response to stimulus", all constituting a high proportion at different time points in shoots and roots, accounting for 18.9 %–28.9 %, 14.4 %–17.8 %, 5.5 %–10.8 %, 6 %–10.8 %, 3.3 %–4.3 %, and 3.3 %–4.3 % of total DEGs, respectively. The GO terms "metabolic process" and "cellular process" were enriched in both shoots and roots at different time points. According to the "cellular component" classification, DEGs that mapped to "cell", "cell part", and "organelle" constituted a high proportion at different time points in shoots and roots, accounting for 35 %–49.2 %, 35 %–49.2 %, and 29.1 %–41.3 % of total DEGs, respectively. The GO terms "membrane-enclosed lumen" was enriched in 12S and 12R. According to the "molecular function" classification, DEGs were mainly mapped to "binding" and "catalytic activity" at different time points in shoots and roots, accounting for 19.5 %–21 % and 22.1 %–34.1 % of total DEGs, respectively. The GO terms "electron carrier activity", "protein binding transcription factor activity", and "receptor activity" were only enriched in 12S.

4.3. Roots and shoots exhibit spatio-temporal specificity following exposure to low NH_4^+ following N starvation

To understand the biological function of these DEGs and how the DEGs interact, we then employed KEGG enrichment analysis to classify the crucial biological processes and cardinal signaling pathways. Our analysis shows that DEGs involved in amino acid metabolism, carbohydrate metabolism, and phenylpropanoid biosynthesis (especially flavonoids) were all up-regulated in 4R and 4S (Fig. 3). However, the DEGs functioning in plant-pathogen interaction and hormone-signaling pathways were only obviously regulated in 4R (Fig. 4). The transcripts in the plant-pathogen interaction category were up-regulated, followed by hormone-signal transduction, in particular the pathway involving MAPK (mitogen-activated protein kinases) signaling (Supplementary Excel S1). Furthermore, the transcription levels of DEGs related to circadian rhythm also increased in both 4R and 4S (Supplementary Excel S2). With prolonged supplementation, the transcripts related to circadian rhythm were up-regulated more than 2-fold in 12S and 12R, accompanied by increased transcription of flavonoid biosynthesis, especially in 12S (Fig. 4). By contrast, the up-regulated DEGs involved in plant-pathogen interaction and hormone-signaling pathways in 4R were less induced in both 12R and 12S (Fig. 4; Supplementary Excel S2-S4). Furthermore, with increasing time of low NH_4^+ imposition, a set of transcripts encoding enzymes in amino acid and carbohydrate biosynthesis/metabolism were significantly induced in 12S and 12R (Fig. 3 and Supplementary Excel S2). In addition, genes involved in brassinosteroid biosynthesis were up-regulated only in roots, 4R and 12R.



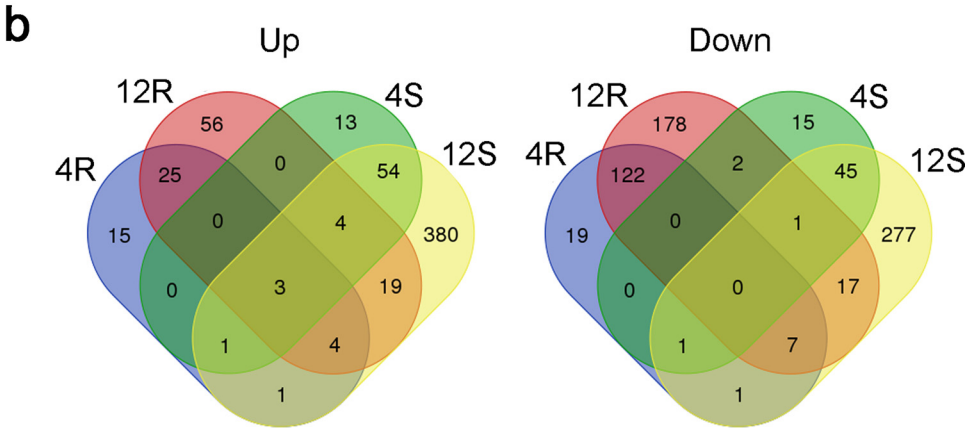


Fig. 2. Numbers and Venn diagram display of DEGs in rice shoots and roots under low-NH_4^+ supplementation following N starvation compared to N starvation (0 N). (a) DEG number in shoots and roots at 4 h and 12 h. (R value < 0.05; genes with the ratio log ≥ 2 or ≤ -2 were selected). Green: down-regulated. Red: up-regulated. **(b)** Venn diagram shows the up-regulated and down-regulated number of DEGs in shoots and roots at different time points. (> 2-fold) (Blue, Pink, Green and Yellow indicates 4S, 12S, 4R, and 12R, respectively). 4S: 4 -h-Shoot; 4R: 4 -h-Root; 12S: 12 -h-Shoot; 12R: 12 -h-Root (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

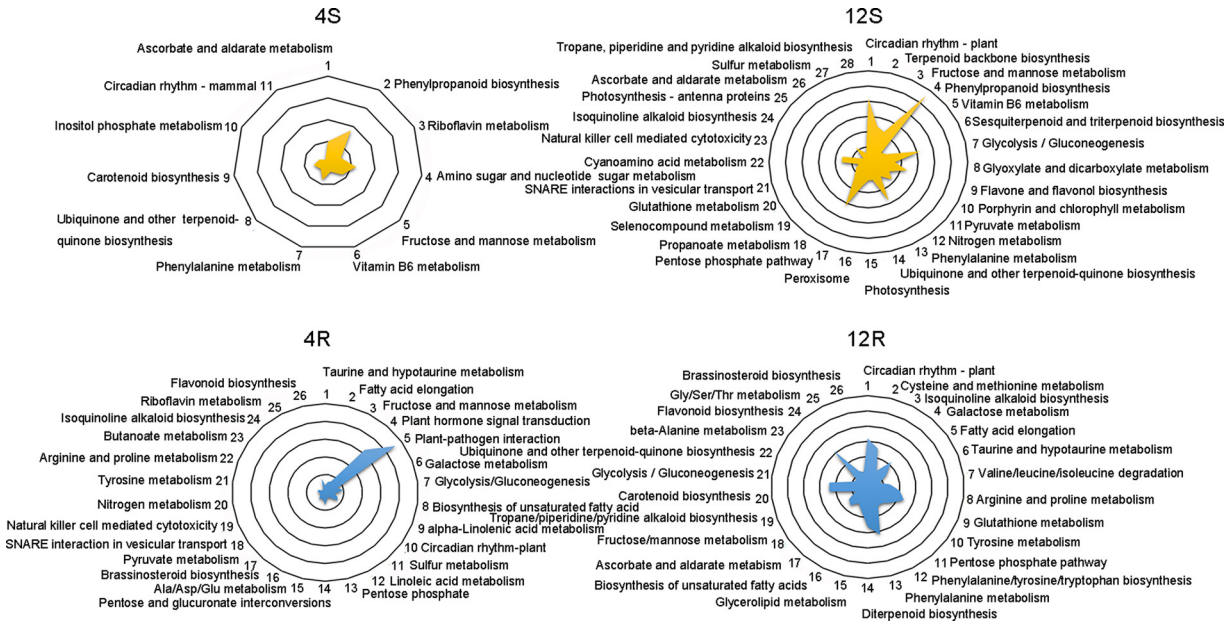


Fig. 3. Spatio-temporal specificity in roots and shoots under low-NH_4^+ supplementing following N starvation. Up-regulated metabolic pathways were selected as follows: R value < 0.05; genes with the regulation ratio log ≥ 2 or ≤ -2 were selected. 4S: 4 -h-Shoot; 4R: 4 -h-Root; 12S: 12 -h-Shoot; 12R: 12 -h-Root.

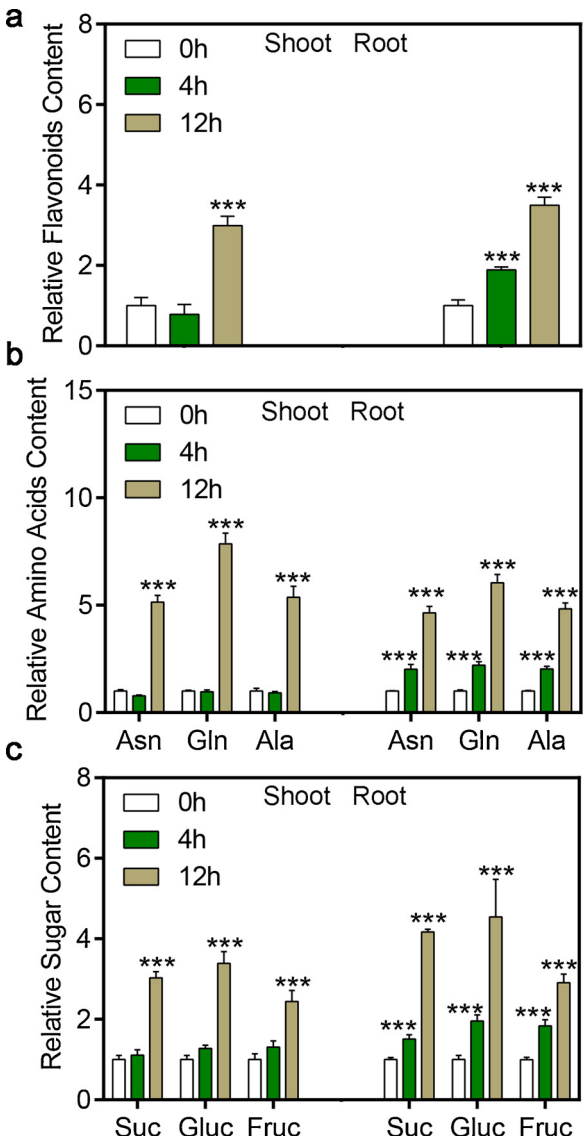


Fig. 4. Flavonoid, sugar, and amino acid analyses in shoots and roots under low-NH_4^+ supplementation following N starvation. (a) flavonoids, **(b)** sugars (sucrose, fructose, and glucose) and **(c)** amino acids (asparagine, glutamine, and alanine) were analyzed at 4 h and 12 h in shoots and roots under low 0.2 mM NH_4^+ supplementation following N starvation. Values are means ± SD (n = 3). *** indicate significant difference at $P < 0.01$.

Further, we measured the contents of flavonoids, sugars (sucrose, fructose and glucose), and amino acids in shoots and roots at different time points (Fig. 4; Table S3). Consistent with RNA-seq results, the contents of flavonoids, sugars, and amino acids were altered 0.8, 1.1–1.3, and 0.8–1.8-fold in 4S, and 1.9, 1.5–1.9, and 2.1–14.8-fold in 4R, compared with the N-starvation (0 N) control. With prolonged supplementation, the contents of flavonoids, sugars, and amino acids were increased 2.9, 2.6–3.1, 2.4–8.7-fold in 12S, and 3.5, 2.9–4.5, and 5–50.1-fold in 12R compared to the N-starvation (0 N) control.

4.4. TFs (transcription factors) in hormone regulation in response to low NH_4^+ following N starvation

Our data show that 26 and 33 TFs were regulated in 4R and 12R, respectively, while only a few TFs were regulated in the shoot (3 TFs and 15 TFs in 4S and 12S, respectively) (Fig. 5; Supplemental Excel S1-S4). TFs also exhibited spatio-temporal specificity under 0.2 mM ammonium, indicating that 7 TFs were only induced in 12R (Supplemental Excel S4). Likewise, 2 TFs were only induced in 4S, while 14 were solely expressed in 12S. TFs uncovered mainly belonged to the TIFY/ZIM (zinc- finger protein expressed in inflorescence meristem), bHLH (*basic helix-loop-helix protein factor*), MYB (*MYB domain protein*) and WRKY (*WRKY DNA-binding protein*) families. We then further analyzed the transcriptional regulatory network of TFs in roots. The analysis showed that most low-NH_4^+ responsive genes, covering plant-pathogen interaction, amino acid biosynthesis and metabolism, and flavonoid biosynthesis, might be bound by one or more TFs (Fig. 5). With prolonged treatment time, the TFs and responsive genes were all increased (Fig. 5b). Of them, *TIFY/ZIM* genes (*OsJAZ9/OsTIFY11a* to *OsJAZ12/OsTIFY11d*), including a JA ZIM-domain (Jasmonate Zim Domain), were up-regulated under 0.2 mM ammonium in both 4R and 12R, especially OsJAZ9, which was most pronouncedly induced by low NH_4^+ in 4R and 12R. Hence, it was important to investigate whether these genes function in response to low NH_4^+.

4.5. Upregulation of OsJAZ9 resulted in increased NH_4^+ acquisition and root growth under low NH_4^+ following N starvation

RNA-seq results show that OsJAZ9 was induced in roots after 4 h and 12 h of low-NH_4^+ treatment (Fig. 5). To further investigate the function of *OsJAZ9* in response to low NH_4^+, *OsJAZ9* overexpression (JAZ9*ox*) material and its background (ZH11) were used. Following imposition of 0.2 mM NH_4^+ after N starvation, the shoot displayed minor differences, but root growth in JAZ9*ox* increased remarkably compared with the wild type ZH11 (Fig. 6a–d). Compared with the N-starvation (0 N) control, the total root length, area, and volume were increased 29.2 %, 43.2 %, 120.5 % in ZH11 under re-supply 0.2 mM NH_4^+ condition, however they were 183.2 %, 281.1 %, 386.9 % in JAZ9*ox*, suggesting an OsJAZ9 function in promoting root growth in response to low NH_4^+ treatment (Fig. 6a–d). Considering the transcript induction of amino acid and carbohydrate metabolic genes in the low-NH_4^+ condition, we then analyzed amino acid and sugar accumulation in ZH11 and JAZ9*ox* (Fig. 3 and Fig. 6e-f). Our data show that sugar and amino acids contents were all increased in JAZ9*ox* compared with ZH11 after re-supply of 0.2 mM NH_4^+ (Fig. 6e-f). Furthermore, our ^{15}N-isotope labeling results revealed that both ZH11 and JAZ9*ox* had higher excess atom% ^{15}N and/or N_{dff} % ^{15}N in tissue under 0.2 mM ammonium compared with the N-starvation (0 N) control in roots after 4 h of treatment, while JAZ9*ox* increased 41.4-fold in atom% ^{15}N compared with a 12.1-fold change in ZH11 under 0.2 N (Fig. 6g-h). Taken together, our results suggest that OsJAZ9 plays a positive role in shoot and root growth, especially in roots, under N starvation followed by re-supply of low NH_4^+, which results in the promotion of N absorption and enhanced sugar and amino acid biosynthesis.

To further test the potential regulatory role of OsJAZ9, we chose *OsCOI1*-RNAi (*coi1*), which, when knocked down, results in higher accumulation of OsJAZ9 *in situ* (Chini et al., 2009; Yang et al., 2012). Consistent with this, our results show that *coi1* possesses stronger root growth promotion compared with its background ZH11 under low NH_4^+ re-supply after N starvation (Fig. 7a-b). Under low-NH_4^+ re-supply, the increase in total root length, root area, and root volume in the *coi1* mutant was 1.1, 1.3, 1.5 times that of ZH11 (Fig. 7c-e). The ^{15}N-isotope labeling data show that atom% ^{15}N and/or N_{dff} % ^{15}N increased 8.9-fold in ZH11, and 10.9-folds in the *coi1* mutant after 4 h of treatment. These data clearly show that OsJAZ9 positively regulates NH_4^+ acquisition under re-supply of low NH_4^+ following N starvation.

5. Discussion

N is necessary for plant growth. However, high N-application rates can, in fact, lead to decreased grain yields, and to drastic declines in nitrogen-use efficiency (NUE), with the additional consequence of increased N loss to the environment, polluting both the atmosphere and water systems (Chen et al., 2013; Coskun et al., 2017). Thus, the

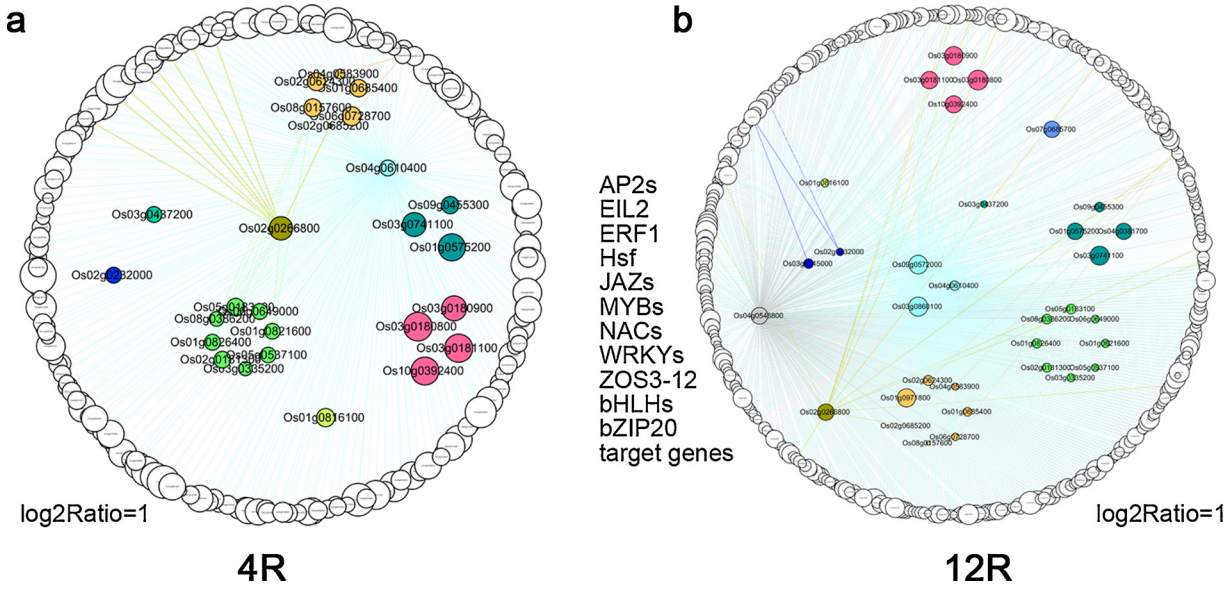


Fig. 5. The regulatory network for low-NH_4^+-induced TFs in roots. Regulatory network involving 26 TFs in 4R **(a)** and 33 TFs in 12R **(b)**. White colour indicates putative downstream genes, other colours indicate TFs, and the same colour refer to the same protein family (q value < 0.05, score > 20). The circle size indicated the log2Ratio. 4R: 4 -h-Root; 12R: 12 -h-Root.

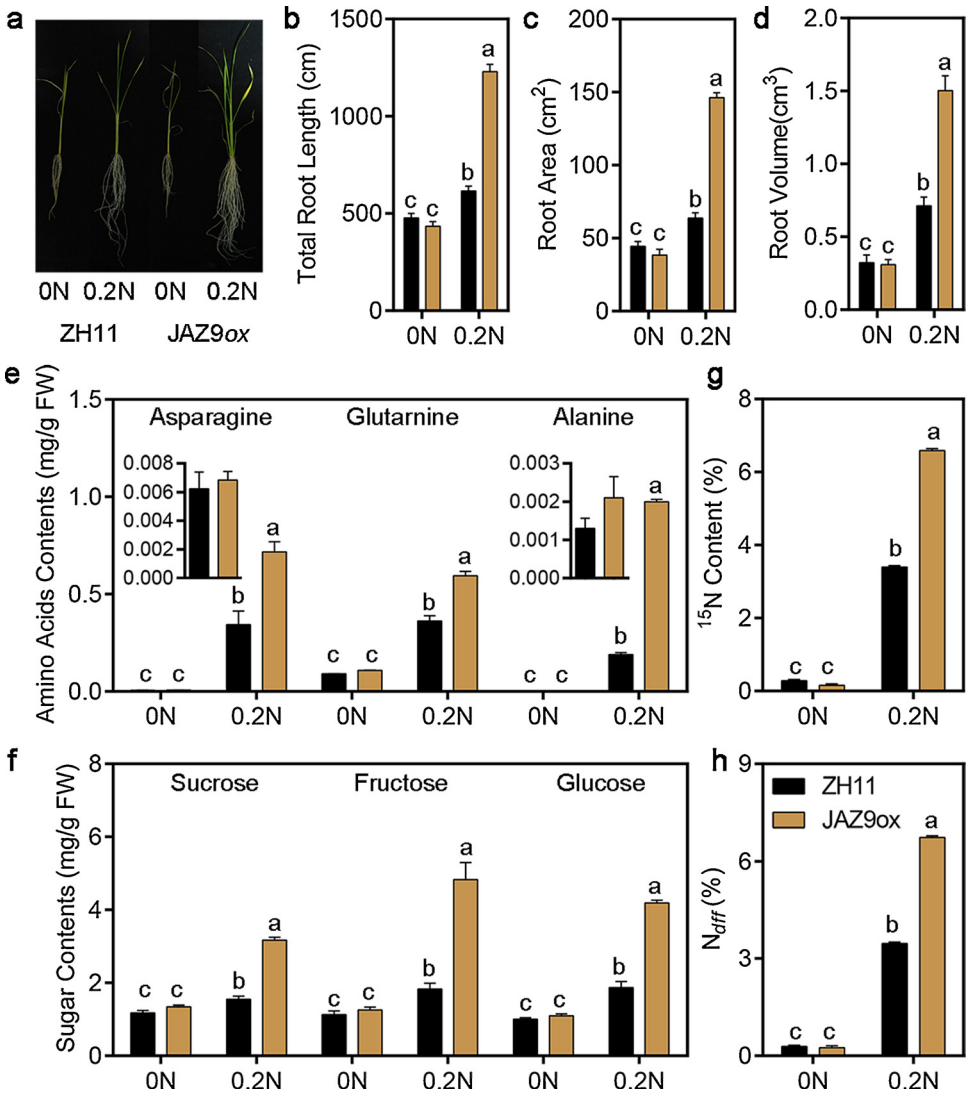


Fig. 6. OsJAZ9 reprograms metabolic processes in acclimation to low-NH_4^+ resupply following N starvation. (a) Phenotypes of JAZ9ox grown with resupply of 0 (0 N) and 0.2 mM ammonium (0.2 N) following N starvation; total root length **(b)**; root surface area **(c)**; and root volume **(d)** of ZH11 and JAZ9ox grown under resupply of 0 (0 N) and 0.2 mM ammonium (0.2 N) following N starvation; contents of sugars (sucrose, fructose, and glucose) **(e),** and amino acids (asparagine, glutamine, and alanine) **(f)** were analysed at 12 h in ZH11 and JAZ9*ox* roots under 0 (0 N) and 0.2 mM NH_4^+ (0.2 N) supplementation following N starvation; excess atom% ^{15}N **(g)** and N_{diff} % ^{15}N **(h)** in ZH11 and JAZ9*ox* roots under 0 (0 N) and 0.2 mM NH_4^+ (0.2 N) supplementation following N starvation. For (b-d), values are means ± SD (n = 30); for (e-f), values are means ± SD (n = 3). Data were analyzed by two-way ANOVA following Duncan's test. Error bars with different letters represent a statistical difference (P < 0.05, Duncan's test).

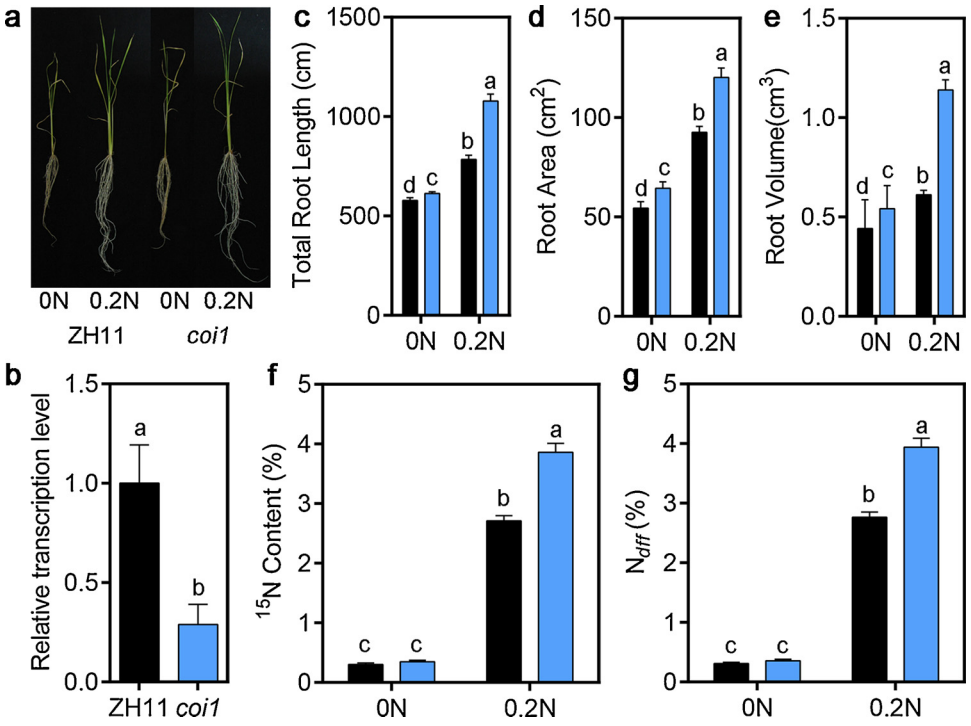


Fig. 7. *coi1* mutant improves acclimation to low-NH_4^+ re-supply following N starvation. **(a)** Phenotypes of *coi1* grown with 0 (0 N) and 0.2 mM ammonium (0.2 N) following N starvation; relative transcription of *OsCOI1* in ZH11 and *coi1* mutants **(b)**; total root length **(c)**; root surface area **(d)**; and root volume **(e)** of ZH11 and JAZ9**ox** grown with 0 (0 N) and 0.2 mM ammonium (0.2 N) following N starvation ; excess atom% ^{15}N **(f)** and N_{dff} % ^{15}N **(g)** in ZH11 and JAZ9*ox* roots under 0 (0 N) and 0.2 mM NH_4^+ (0.2 N) supplementation following N starvation. For (c-e), values are means ± SD (n = 30); for (b, f-g), values are means ± SD (n = 3). Data were analyzed by two-way ANOVA following Duncan's test. Error bars with different letters represent a statistical difference ($P < 0.05$, Duncan's test).

development of cultivars and practices that can make the best use of nitrogen in soil under low-nitrogen conditions, in particular for the world's leading grain crops, is essential for the sustainability of agriculture (Glass et al., 2002; Shi et al., 2010). The RNA-seq analysis is an efficient method to study genome-wide changes in gene transcription and to screen existing gene resources in response to different N concentrations (Balazadeh et al., 2014; Sun et al., 2017; Yang et al., 2015). Previous studies identified responsive genes by two approaches: One involves reduction and/or depletion of N in the growth media, aimed to investigate low-N stress (N starvation); the other is achieved by re-supplying normal N to seedlings grown in media with no or little N, focused on NUE (N supplementation). Key genes functioning in plant survival were involved in response to N starvation, including this involved in the overall stress response, chlorophyll synthesis, and N assimilation (Chandran et al., 2016; Sun et al., 2017; Yang et al., 2015). Furthermore, genes related to N absorption and assimilation were up-regulated during N supplementation (Chandran et al., 2016; Sun et al., 2017; Yang et al., 2015). However, these two approaches have not been effective in investigating the response to low NH_4^+ supply. Here, we used N-starved seedlings subsequently exposed to low NH_4^+ (0.2 mM NH_4^+) at different time points (0 h, 4 h and 12 h), and then analyzed the transcriptomic profiles in shoots and roots. Our study exposits the global regulation of genes transcription and metabolic processes in response to the low-NH_4^+ signal.

5.1. Spatio-temporal specificity of genes expression in rice under low-NH_4^+ resupply after N starvation

When seedlings grown in N-free conditions are transferred to low NH_4^+, the root system responds rapidly to the nutritional signal, and transient and specific expression of NH_4^+-responsive genes is evident at the genome-wide level (Fig. 3). In our study, we investigated DEGs in response to low NH_4^+ supplementation, and results show that 138 (75 up-regulated /63 down-regulated) and 597 (179 up-regulated /418 down-regulated) genes are differentially expressed in rice shoots and roots after 4 h of low-NH_4^+ exposure compared to control (Fig. 2). This phenomenon implies that the rice root inherited a complex responsive network in dealing with low NH_4^+ supplementation. Based on GO enrichment analysis, most of the DEGs were distributed among "cellular process", "metabolic process", "binding", "catalytic activity", and "cellular process" (Fig. S1). This indicates that rice initiates complex and broad metabolic processes to respond to low NH_4^+ supplementation. Notably, as one might expect, genes encoding membrane and transporter activity are also involved in the low-NH_4^+ response (Fig. 3). In addition, the GO terms "plant hormones signal transduction" and "plant-pathogen interaction" were only induced in 4R, indicating these two processes play critical roles in the early response to low NH_4^+ in roots. Previous studies revealed that auxin, cytokinin, and abscisic acid (ABA) have critical roles in the plant response to fluctuating availability of useable N (Kiba et al., 2011; Krapp et al., 2011; Sakakibara et al., 2006). Here, hormone-related transcripts, in particular those pertaining to the JA signaling pathway, were up-regulated significantly at 4R and magnified at 12R (Supplementary Excel S2 and S4). Interestingly, genes involved in BR biosynthesis, which can promote root growth, were only induced in 4R and 12R (Kim et al., 2007; Yun et al., 2009) (Fig. 3). Still, we also identified three genes, DR8 (Os09g0343200), and two uncharacterized genes (Os10g0506900 and Os07g0529600) that were common induced in 4R, 4S, 12R and 12S, indicating that these genes are involved in the synergistic regulation of shoots and roots during the low-NH_4^+ response (Fig.2). Interestingly, DR8 has been reported to possess a dual function in disease resistance and thiamine accumulation (Wang et al., 2006). These findings suggest that low-NH_4^+ signaling may interconnect with defense signaling, and that identification of the function of DR8 in the low-NH_4^+ response might provide useful information for future studies on the intersection of nutrient and defense signaling pathways in plants (Wang et al., 2006). Furthermore, we compared the regulated genes under high-NH_4^+ (Sun et al., 2017) and low-NH_4^+ conditions and found 98 (59 up-regulated /39 down-regulated) and 117 (20 up-regulated /97 down-regulated) genes to be commonly regulated in rice shoots and roots after 4 h/12 h of low-NH_4^+ and high NH_4^+ exposure compared with control (Fig. S3 and Table S4). Of these common genes, several expressed enzymes, transporters, and transcription factors, indicating that rice might engage similar metabolic processes and transcriptional regulatory pathways under low NH_4^+ and high NH_4^+.

In our study, low NH_4^+ supplementation following N starvation was

still not adequate for normal growth in the early stages of resupply, so seedlings remaining under a low-NH_4^+ setting were expected to remain responsive to the "low-NH_4^+ signal". In this state, the plant must adjust whole-plant growth and then utilize the limited N resource. Furthermore, the most important adjustment in plant adaptation to nutrient deficiency is root architecture (Clowes, 1970; Parsons and Sunley, 2001; Ratner, 1945; Zelazny and Vert, 2014). However, knowledge of the role of hormones and associated TFs participating in the response to low NH_4^+ is still limited. Here, increased root length of rice under low NH_4^+ was in agreement with many earlier reports (Singh et al., 2015; Zhang et al., 2015). Consistent with this, 33 hormone-related TFs were up-regulated, and most up-regulated genes were concentrated in the root (Supplementary Excel 3). Furthermore, these TFs primarily belong to the TIFY/ZIM and bHLH families involved in JA signaling (Fig. 5). To date, 15 JAZ proteins have been identified in rice, and many of these genes are involved in nutrient deficiencies (Singh et al., 2015). Hakata et al. (2012) reported that overexpression of *OsJAZ10* led to higher tissue levels of the carbohydrate assimilates. 167 members of the OsbHLH family have been identified in rice, and some of them are related to stress responses, such as *OsbHLH1,* which is involved in cold stress, *RERJ1* and *OsbHLH148,* which are involved in drought stress (Song et al., 2011). This indicates a significant role of JA signaling in the low-NH_4^+ response in the rice root. In addition, *ERF* genes (*OsERF15*, *OsERF1B,* and *OsERF2*), involved in ethylene signaling, were up-regulated as well (Supplementary Excel 3); *ERF*s are involved in the regulation of various stress responses and the expression of stress-related genes to control plant metabolism, growth, and development (Kazan, 2015; Xiao et al., 2016). *OsERF2* was shown to regulate the accumulation of sucrose and UDPG, exerting a significant regulatory role in rice root growth (Xiao et al., 2016). Recently, ethylene was reported to participate in regulation of root hair elongation when Arabidopsis seedlings were exposed to low NH_4^+ (Zhu et al., 2016). Moreover, JA and ethylene, often acting cooperatively, play essential roles in regulating plant defense responses (Kazan, 2015). Lorenzo et al. (2003) suggested that *ERF1* integrates signals from ethylene and JA pathways in the context of plant defense. Thus, JA and ethylene signaling might jointly coordinate the adaptive process under low NH_4^+ in rice. However, whether these genes were uniquely regulated by a "low-NH_4^+" signal rather than a "low-N" signal is still unclear. So, future studies will have to be designed to screen genes regulated by low concentrations of NO_3^- and other N sources, to firmly disentangle differences between responses that are specific to NH_4^+.

5.2. The biosynthesis and metabolism of flavonoid, amino acids, and sugars are important in rice under low NH_4^+ following N starvation

Flavonoids play key roles as signaling molecules, through their ability to interact with a wide range of protein kinases, including MAPK, which regulate key steps of cell growth and differentiation, and response to changing environmental conditions (Brunetti et al., 2013; Buer et al., 2010, 2007). In our study, the GO terms related to flavonoid biosynthesis were upregulated in 4R, 12R, and 12S (Fig. S1). Consistent with this, the flavonoid content increased at 4R and 12R, while an increasing flavonoid content in shoots was only found at 12S, indicating a key role of flavonoids in the signal transduction from roots to shoots (Fig. 3a). Furthermore, phenylpropanoid synthesis, especially flavonoid synthesis, is also responsive to JA, suggesting that JA may function upstream to flavonoids in the low-NH_4^+ response (Buer et al., 2007; Pourcel et al., 2013). In addition, the GO term involved in flavonoids was also up-regulated under NH_4^+-free conditions, underscoring the important role of flavonoids in the NH_4^+-response process, especially in the low-NH_4^+ response (Yang et al., 2015).

Upon low-NH_4^+ supplementation, N-starved seedlings showed transient expression of NH_4^{+-} uptake and N-assimilation genes at both mRNA and protein levels. Here, we determined that two known high-affinity NH_4^+ transporter genes, AMT1.2 and AMT1.3, were induced in roots, revealing that plants began to adapt to the NH_4^+ supplement by increasing NH_4^+ uptake (Supplementary Excel 1). In addition, we also identified that five N-metabolism-related early nodulin genes, Os06g014220, Os06g0142350, Os06g0142400, Os06g0142350, and Os06g0142200, were up-regulated in roots (Supplemental Excel S2 and S4). We also found multiple GO terms functioning in "amino acid biosynthesis/metabolism" and "nitrogen metabolism" were significantly up-regulated, indicating that increased conversion of inorganic N into organic N products, such as amino acids, was critical. Elevated levels of amino acids in plant cells have been correlated with improved environmental adaptation, aiding in plant water relations and in protecting key enzymes (Flowers and Colmer, 2015). RNA-seq results suggest that 1, 2, 2 and 8 GO terms were involved in amino acid biosynthesis/metabolism, with Gln, aspartate (Asp), threonine (Thr), and aromatic amino acids being predominant (Fig. S1). Accordingly, we found Asn, Gln, Thr, and aromatic amino acids increased at 4R and then continuously increased up to 12R (Fig. 4b; Table S3). Meanwhile, these amino acids were also elevated at 12S (Fig. 4b; Table S3). Furthermore, inorganic N in plants is assimilated initially to asparagine (Asn) and glutamine (Gln), and these amino acids serve as important N carriers (Alexova et al., 2015; Pratelli and Pilot, 2014). Aromatic amino acids are not only synthesized into protein but also act as precursors for secondary metabolites (Tzin and Galili, 2010). Thr might relate to plant defenses or serve as a precursor for other amino acids such as isoleucine (Balazadeh et al., 2014). This reveals that amino acids metabolism plays an important role under low-NH_4^+ supplementation after N starvation. These observations are in agreement with reports that amino acids production is stimulated under shifting N-utilization conditions (Chandran et al., 2016; Pratelli and Pilot, 2014).

Previous studies suggested that activation of amino acid and storage protein synthesis was metabolically regulated and stimulated by carbohydrate feeding and/or supply of carbon precursors (Lemoine et al., 2013). Similarly, increased N supplementation also stimulated carbohydrate biosynthesis and metabolism. Our data show that the GO terms involved in sugar metabolism were enriched in all treatments. Sucrose, fructose, and glucose all increased at 4R and then continuously increased up to 12R. Meanwhile, these three sugars were also raised at 12S (Fig. 4c). Previous genome-wide transcriptome data showed that, whether seedlings were shifted from a normal environment to limiting N conditions or from N starvation to re-supply of normal N, the GO term of carbon/nitrogen metabolism was induced, indicating the fundamentally important nature of plastic C/N metabolism pathway adjustment in response to varying N conditions (Moorhead and Smith, 2003; Perchlik and Tegeder, 2018; Raab and Terry, 1995).

6. The transcription factor OsJAZ9 mediates metabolic reprogramming during re-supply of low NH_4^+ following N starvation in rice

RNA-seq is an effective method to investigate the regulation of genome-wide transcriptome in responses to varying N conditions and environmental stresses (Balazadeh et al., 2014; Sun et al., 2017; Yang et al., 2015). To test with RNA-seq analysis in response to low NH_4^+, we chose a transcription factor, OsJAZ9, which was obviously up-regulated in 4R and 12R (Fig. 5). OsJAZ9 was an important TF involved in JA signaling pathway, and was induced by N, P, K, Zn, and Fe deficiency (Singh et al., 2018; Wu et al., 2015). The mutant with over-expressed *OsJAZ9* exhibited faster growth under K^+ deficiency and higher tolerance to salt stress by regulating K^+ transport, suggesting an important role of OsJAZ9 in response to nutritional deficiency and environmental stress (Singh et al., 2018). Here, we demonstrate that OsJAZ9 might be the major TF in flexibly adjusting growth in response to low NH_4^+ supplementation, to match anticipated changes in resource availability after N starvation, in rice. Our data show that low-NH_4^+ supplementation prompts root growth in both ZH11 and JAZ9ox, and that JAZ9ox exhibits enhanced root growth (Fig. 6b-d).

Accordingly, we found that sugar, amino acids, and NH_4^+ absorption in JAZ9*ox* under low NH_4^+ were all increased (Fig. 6). Consistently, *coi1* mutant, whose mutation results in OsJAZ9 protein accumulation *in situ*, also exhibited root growth promotion and then higher NH_4^+ absorption under low NH_4^+ following N starvation (Fig. 7). Previous studies demonstrated that JAZ proteins promote growth and reproductive success at least in part by metabolic effects inherent in an adaptive response under stress (Seo et al., 2011; Wu et al., 2015). Hence, we conclude that JA signaling involving *OsJAZ9* plays a key role in rice in the response to low NH_4^+ through regulating sugar biosynthesis, amino acid biosynthesis, and absorption of NH_4^+.

7. Conclusions

In summary, RNA-seq analysis allowed us to obtain whole-genome transcriptional regulation and processes potentially implicated in the early response to low NH_4^+ supplementation following N starvation. Our RNA-seq data indicate that JA and flavonoids are important signaling molecules in response to low NH_4^+ in roots, and that the genes required in amino acid and carbohydrate metabolism are up-regulated under low-NH_4^+ resupply conditions. Moreover, OsJAZ9 is a critical TF in response to low NH_4^+ and promotes NH_4^+ absorption, sugar and amino acid accumulation, and root growth. More detailed experimental exploration will be needed in order to investigate the function of the genes involved in the low-NH_4^+ response. Clearly, the role of NH_4^+ as a signal to stimulate root growth and enhanced nutrition absorption warrants further study in the future.

Authors' contributions

LS, DWD and WMS conceived and designed the research; LS, DWD and GJL performed the experiments; DWD, LS, YLL, HJK and WMS wrote the manuscript. All authors read and approved the final manuscript.

Funding

This research was financially supported by the National Natural Science Foundation of China (31430095, 31572205 and 31872957), and the University of Melbourne.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank Prof. Zuhua He (Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences) for providing oscoil mutant and Pro. Lizhong Xiong (National Key Laboratory of Crop Genetic Improvement, National Center of Plant Gene Research, College of Life Science and Technology, Huazhong Agricultural University) for providing *OsJAZ9/OsTIFY11a*- OE material.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jplph.2020.153137.

References

Balazadeh, S., Schildhauer, J., Araujo, W.L., Munne-Bosch, S., Fernie, A.R., Proost, S., et al., 2014. Reversal of senescence by N resupply to N-starved *Arabidopsis thaliana*: transcriptomic and metabolomic consequences. J. Exp. Bot. 65, 3975–3992.

Bao, A., Liang, Z., Zhao, Z., Cai, H., 2015. Overexpressing of OsAMT1.3, a high affinity ammonium transporter gene, modifies rice growth and carbon-nitrogen metabolic status. Int. J. Mol. Sci. 16, 9037–9063.

Brunetti, C., Di Ferdinando, M., Fini, A., Pollastri, S., Tattini, M., 2013. Flavonoids as antioxidants and developmental regulators: relative significance in plants and humans. Int. J. Mol. Sci. 14, 3540–3555.

Buer, C.S., Muday, G.K., Djordjevic, M.A., 2007. Flavonoids are differentially taken up and transported long distances in Arabidopsis. Plant Physiol. 145, 478–490.

Buer, C.S., Imin, N., Djordjevic, M.A., 2010. Flavonoids: new roles for old molecules. J. Integr. Plant Biol. 52, 98–111.

Chandran, A.K.N., Priatama, R.A., Kumar, V., Xuan, Y.H., Je, B.I., Kim, C.M., et al., 2016. Genome-wide transcriptome analysis of expression in rice seedling roots in response to supplemental nitrogen. J. Plant Physiol. 200, 62–75.

Chen, G., Guo, S.W., Kronzucker, H.J., Shi, W.M., 2013. Nitrogen use efficiency (NUE) in rice links to NH_4^+ toxicity and futile NH_4^+ cycling in roots. Plant Soil 369, 351–363.

Chini, A., Boter, M., Solano, R., 2009. Plant oxylipins: COI1/JAZs/MYC2 as the core jasmonic acid-signalling module. FEBS J. 276, 4682–4692.

Clowes, F.A., 1970. Nutrition and the quiescent centre of root meristems. Planta 90, 340–348.

Cock, P.J.A., Fields, C.J., Goto, N., Heuer, M.L., Rice, P.M., 2010. The Sanger FASTQ file format for sequences with quality scores, and the Solexa/Illumina FASTQ variants. Nucleic Acids Res. 38, 1767–1771.

Coskun, D., Britto, D.T., Shi, W.M., Kronzucker, H.J., 2017. How plant root exudates shape the nitrogen cycle. Trends Plant Sci. 22, 661–673.

de Jong, M., George, G., Ongaro, V., Williamson, L., Willetts, B., Ljung, K., et al., 2014. Auxin and strigolactone signaling are required for modulation of Arabidopsis shoot branching by nitrogen supply. Plant Physiol. 166, U384–U549.

del Campo, C.P., Garde-Cerdán, T., Sánchez, A.M., Maggi, L., Carmona, M., Alonso, G.L., 2009. Determination of free amino acids and ammonium ion in saffron (*Crocus sativus* L.) from different geographical origins. Food Chem. 114, 1542–1548.

Di, D.W., Sun, L., Zhang, X.N., Li, G.J., Kronzucker, H.J., Shi, W.M., 2018. Involvement of auxin in the regulation of ammonium tolerance in rice (*Oryza sativa* L.). Plant Soil 432, 373–387.

Flowers, T.J., Colmer, T.D., 2015. Plant salt tolerance: adaptations in halophytes. Ann. Bot. 115, 327–331.

Glass, A.D.M., Britto, D.T., Kaiser, B.N., Kinghorn, J.R., Kronzucker, H.J., Kumar, A., et al., 2002. The regulation of nitrate and ammonium transport systems in plants. J. Exp. Bot. 53, 855–864.

Good, A.G., Shrawat, A.K., Muench, D.G., 2004. Can less yield more? Is reducing nutrient input into the environment compatible with maintaining crop production? Trends Plant Sci. 9, 597–605.

Goossens, J., Fernandez-Calvo, P., Schweizer, F., Goossens, A., 2016. Jasmonates: signal transduction components and their roles in environmental stress responses. Plant Mol. Biol. 91, 673–689.

Gruber, N., Galloway, J.N., 2008. An earth-system perspective of the global nitrogen cycle. Nature 451, 293–296.

Hakata, M., Kuroda, M., Ohsumi, A., Hirose, T., Nakamura, H., Muramatsu, M., et al., 2012. Overexpression of a rice TIFY gene increases grain size through enhanced accumulation of carbohydrates in the stem. Biosci. Biotech. Bioch. 76, 2129–2134.

Kazan, K., 2015. Diverse roles of jasmonates and ethylene in abiotic stress tolerance. Trends Plant Sci. 20, 219–229.

Khan, M.I.R., Trivellini, A., Fatma, M., Masood, A., Francini, A., Iqbal, N., et al., 2015. Role of ethylene in responses of plants to nitrogen availability. Front. Plant Sci. 6.

Kiba, T., Kudo, T., Kojima, M., Sakakibara, H., 2011. Hormonal control of nitrogen acquisition: roles of auxin, abscisic acid, and cytokinin. J. Exp. Bot. 62, 1399–1409.

Kim, T.W., Lee, S.M., Joo, S.H., Yun, H.S., Lee, Y., Kaufman, P.B., et al., 2007. Elongation and gravitropic responses of Arabidopsis roots are regulated by brassinolide and IAA. Plant Cell Environ. 30, 679–689.

Krapp, A., Berthome, R., Orsel, M., Mercey-Boutet, S., Yu, A., Castaings, L., et al., 2011. Arabidopsis roots and shoots show distinct temporal adaptation patterns toward nitrogen starvation. Plant Physiol. 157, 1255–1282.

Kronzucker, H.J., Glass, A.D.M., Siddiqi, M.Y., 1999. Inhibition of nitrate uptake by ammonium in barley. Analysis of component fluxes. Plant Physiol. 120, 283–291.

Kronzucker, H.J., Glass, A.D.M., Siddiqi, M.Y., Kirk, G.J.D., 2000. Comparative kinetic analysis of ammonium and nitrate acquisition by tropical lowland rice: implications for rice cultivation and yield potential. New Phytol. 145, 471–476.

Lemoine, R., La Camera, S., Atanassova, R., Deedaldeechamp, F., Allario, T., Pourtau, N., et al., 2013. Source-to-sink transport of sugar and regulation by environmental factors. Front. Plant Sci. 4.

Lorenzo, O., Piqueras, R., Sanchez-Serrano, J.J., Solano, R., 2003. ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate pathways in plant defense. Plant Cell 15, 165–178.

Min, J., Zhang, H.L., Shi, W.M., 2012. Optimizing nitrogen input to reduce nitrate leaching loss in greenhouse vegetable production. Agric. Water Manage. 111, 53–59.

Moorhead, G.B., Smith, C.S., 2003. Interpreting the plastid carbon, nitrogen, and energy status. A role for PII? Plant Physiol. 133, 492–498.

Ohashi, M., Ishiyama, K., Kojima, S., Konishi, N., Nakano, K., Kanno, K., et al., 2015. Asparagine synthetase 1, but not asparagine synthetase 2, is responsible for the biosynthesis of asparagine following the supply of ammonium to rice roots. Plant Cell Physiol. 56, 769–778.

Ordonez, A.A.L., Gomez, J.D., Vattuone, M.A., Isla, M.I., 2006. Antioxidant activities of Sechium edule (Jacq.) Swartz extracts. Food Chem. 97, 452–458.

Parsons, R., Sunley, R.J., 2001. Nitrogen nutrition and the role of root-shoot nitrogen signalling particularly in symbiotic systems. J. Exp. Bot. 52, 435–443.

Perchlik, M., Tegeder, M., 2018. Leaf amino acid supply affects photosynthetic and plant nitrogen use efficiency under nitrogen stress. Plant Physiol. 178, 174–188.

Pourcel, L., Irani, N.G., Koo, A.J.K., Bohorquez-Restrepo, A., Howe, G.A., Grotewold, E., 2013. A chemical complementation approach reveals genes and interactions of flavonoids with other pathways. Plant J. 74, 383–397.
Pratelli, R., Pilot, G., 2014. Regulation of amino acid metabolic enzymes and transporters in plants. J. Exp. Bot. 65, 5535–5556.
Quan, X.Y., Zeng, J.B., Ye, L.Z., Chen, G., Han, Z.G., Shah, J.M., et al., 2016. Transcriptome profiling analysis for two Tibetan wild barley genotypes in responses to low nitrogen. BMC Plant Biol. 16.
Raab, T.K., Terry, N., 1995. Carbon, nitrogen, and nutrient interactions in beta-vulgaris L as influenced by nitrogen-source, NO_3^- versus NH_4^+. Plant Physiol. 107, 575–584.
Ranathunge, K., El-kereamy, A., Gidda, S., Bi, Y.M., Rothstein, S.J., 2014. AMT1;1 transgenic rice plants with enhanced NH_4^+ permeability show superior growth and higher yield under optimal and suboptimal NH_4^+ conditions. J. Exp. Bot. 65, 965–979.
Ratner, E.I., 1945. Interaction between roots and soil colloids as a problem of mineral nutrition of plants; role of the root system. Doklady Akademii nauk Soiuza Sovetskikh Sotsialisticheskikh Respublik 48, 64–67.
Sakakibara, H., Takei, K., Hirose, N., 2006. Interactions between nitrogen and cytokinin in the regulation of metabolism and development. Trends Plant Sci. 11, 440–448.
Seo, J.S., Joo, J., Kim, M.J., Kim, Y.K., Nahm, B.H., Song, S.I., et al., 2011. OsbHLH148, a basic helix-loop-helix protein, interacts with OsJAZ proteins in a jasmonate signaling pathway leading to drought tolerance in rice. Plant J. 65, 907–921.
Sharma, M., Laxmi, A., 2016. Jasmonates: Emerging players in controlling temperature stress tolerance. Front. Plant Sci. 6.
Shi, W.M., Xu, W.F., Li, S.M., Zhao, X.Q., Dong, G.Q., 2010. Responses of two rice cultivars differing in seedling-stage nitrogen use efficiency to growth under low-nitrogen conditions. Plant Soil 326, 291–302.
Singh, A.P., Pandey, B.K., Deveshwar, P., Narnoliya, L., Parida, S.K., Giri, J., 2015. JAZ repressors: potential involvement in nutrients deficiency response in rice and chickpea. Front. Plant Sci. 6, 975.
Singh, A.P.P.B., Mehra, P., Chandan, R.K., Jha, G., Giri, J., 2018. OsJAZ9 overexpressing improves potassium deficiency tolerance in rice by modulating jasmonic acid levels and signaling. Plant Biol.
Socolow, R.H., 1999. Nitrogen management and the future of food: lessons from the management of energy and carbon. Proc. Natl. Acad. Sci. U. S. A. 96, 6001–6008.
Song, S.S., Qi, T.C., Huang, H., Ren, Q.C., Wu, D.W., Chang, C.Q., et al., 2011. The jasmonate-ZIM domain proteins interact with the R2R3-MYB transcription factors MYB21 and MYB24 to affect jasmonate-regulated stamen development in *Arabidopsis*. Plant Cell 23, 1000–1013.
Sun, L., Di DW, L.G.J., Kronzucker, H.J., Shi, W.M., 2017. Spatio-temporal dynamics in global rice gene expression (*Oryza sativa* L.) in response to high ammonium stress. J. Plant Physiol. 212, 94–104.
Swarbreck, S.M., Wang, M., Wang, Y., Kindred, D., Sylvester-Bradley, R., Shi, W., et al., 2019. A roadmap for lowering crop nitrogen requirement. Trends Plant Sci. 24, 892–904.
Takehisa, H., Sato, Y., Antonio, B.A., Nagamura, Y., 2013. Global transcriptome profile of rice root in response to essential macronutrient deficiency. Plant Signal. Behav. 8, e24409.
Tzin, V., Galili, G., 2010. New Insights into the shikimate and aromatic amino acids biosynthesis pathways in plants. Mol. Plant 3, 956–972.
Verma, V., Ravindran, P., Kumar, P.P., 2016. Plant hormone-mediated regulation of stress responses. BMC Plant Biol. 16, 86.
Wang, Y.Y., Khoo, K.H., Chen, S.T., Lin, C.C., Wong, C.H., Lin, C.H., 2002. Studies on the immuno-modulating and antitumor activities of *Ganoderma lucidum* (Reishi) polysaccharides: functional and proteomic analyses of a fucose-containing glycoprotein fraction responsible for the activities. Bioorg. Med. Chem. 10, 1057–1062.
Wang, G.N., Ding, X.H., Yuan, M., Qiu, D.Y., Li, X.H., Xu, C.G., et al., 2006. Dual function of rice OsDR8 gene in disease resistance and thiamine accumulation. Plant Mol. Biol. 60, 437–449.
Wu, H., Ye, H., Yao, R., Zhang, T., Xiong, L., 2015. OsJAZ9 acts as a transcriptional regulator in jasmonate signaling and modulates salt stress tolerance in rice. Plant Sci. 232, 1–12.
Xiao, G.Q., Qin, H., Zhou, J.H., Quan, R.D., Lu, X.Y., Huang, R.F., et al., 2016. OsERF2 controls rice root growth and hormone responses through tuning expression of key genes involved in hormone signaling and sucrose metabolism. Plant Mol. Biol. 90, 293–302.
Xuan, Y.H., Priatama, R.A., Huang, J., Je, B.I., Liu, J.M., Park, S.J., et al., 2013. Indeterminate domain 10 regulates ammonium-mediated gene expression in rice roots. New Phytol. 197, 791–804.
Yang, D.L., Yao, J., Mei, C.S., Tong, X.H., Zeng, L.J., Li, Q., et al., 2012. Plant hormone jasmonate prioritizes defense over growth by interfering with gibberellin signaling cascade. Proic. Natl. Acad. Sci. U. S. A. 109 E1192-E200.
Yang, S.Y., Hao, D.L., Song, Z.Z., Yang, G.Z., Wang, L., Su, Y.H., 2015. RNA-Seq analysis of differentially expressed genes in rice under varied nitrogen supplies. Gene 555, 305–317.
Yun, H.R., Joo, S.H., Park, C.H., Kim, S.K., Chang, S.C., Kim, S.Y., 2009. Effects of brassinolide and IAA on ethylene production and elongation in maize primary roots. J. Plant Biol. 52, 268–274.
Zelazny, E., Vert, G., 2014. Plant nutrition: root transporters on the move. Plant Physiol. 166, 500–508.
Zhang, Y.J., Tan, L.B., Zhu, Z.F., Yuan, L.X., Xie, D.X., Sun, C.Q., 2015. TOND1 confers tolerance to nitrogen deficiency in rice. Plant J. 81, 367–376.
Zhu, C.H., Yang, N., Guo, Z.F., Qian, M., Gan, L.J., 2016. An ethylene and ROS-dependent pathway is involved in low ammonium-induced root hair elongation in *Arabidopsis* seedlings. Plant Physiol. Biochem. 105, 37–44.