Internal ammonium excess induces ROS-mediated reaction and causes carbon scarcity in rice

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Abstract

Background: Overuse of nitrogen fertilizers is often applied to satisfy strong nitrogen demand of high-yielding rice, leading to persistent NH_4^+ excess in the plant. However, the mechanisms constraining the effectiveness of elevated plant NH_4^+ in plant growth and grain yield of rice are not sufficiently addressed. In the current study, we attempt to reveal the nature or mode-of-action of such internal NH_4^+ excess in rice, and the efficient coordination measure with current high N fertilizer cropping systems is investigated.

Results: By mimicking a rapid accumulation of plant NH_4^+ and an RNA-Seq analysis, the present work reveals that internal NH_4^+ excess in rice plant initiates a burst of reactive oxygen species (ROS) and triggers probably specifically the activation of glutathione transferase (GST)-mediated glutathione cycling for ROS cleavage. Meanwhile, the suppression of the expression of genes involved in photon capture and the activity of primary CO_2 fixation enzymes (Rubisco), provides implications of a reduction in photosynthetic carbon income. Along the progress of NH_4^+ / ROS stresses, enhanced energy-producing processes (carbon breakdown) take place as indicated by strong induction of glycolysis related genes to meet the demand of energy consuming activation of ROS-cleaving systems. The development of these defensive reactions causes a sugar scarcity in the plant that accumulatively leads to growth inhibition. To the opposite direction, a sucrose feeding treatment to the roots renders the accumulation of free NH_4^+ and ROS, partly restores the activities of photosynthetic CO_2 fixation and thus alleviates the scarcity in active carbon source. **Conclusion:** Our results demonstrate the necessity of efficient carbon coordination, aiming at improving the nitrogen performances under current N fertilizer overuse circumstances.

Background

Nitrogen (N) limitation was a leading constraint on the grain yield of rice [1, 2]. Leaf N accounted for the largest N sink of rice plant, ca. 80% of which was distributed in the chloroplast and stored as Ribulose-1,5-bisphosphate carboxylase/oxygenases (Rubisco), the primary carbon fixation enzymes of C_3 plants [3]. Photosynthesis was tightly correlated with leaf N content [4], and more than 80% of grain N was derived from leaves in rice [5]. Hence, insufficient leaf N storage would lead to reduction of photosynthetic carbon fixation efficiency and was therefore considered as a major limitation to biomass and grain production of cereal ecosystems [6–9].

In China's rice farming, to satisfy the N demand of higher grain yield (6.4 t/hm^2 or above), the average N input was normally over 180 kg/hm^2 [10]. In the high-yielding rice farming areas, the N input could even reach to 300 kg/hm^2 and this was particularly the case for recent super-hybrid rice cultivars that achieved as high as $>10 \text{ t/hm}^2$ of grain yields [11]. In soils, applied N fertilizers (e.g. urea form accounted for the majority of current N fertilizers) would rapidly convert to ammonium with the potent reactions of ureases. And in well-ventilated dryland soils, ammonium could convert to another root available nitrogen form—nitrate through the actions of nitrification microbes. However, in rice paddy soils, ca. 70–80% of the

growth period was water flooded, causing an anaerobic environment that largely prevented the process of nitrification. To this end, retention of higher concentration of ammonium around the roots is a generalized fact in rice paddies. Recent results showed that over-dosed N fertilizers strengthened excessive N accumulation in rice plant that however did not accordingly result in increased grain yields [12]. Therefore, low N use efficiency and its agronomic benefits were major problems of high N input rice farming.

Plant responses to toxic concentrations of NH_4^+ supplied exogenously had been extensively demonstrated centering the (molecular) mechanisms involved in the configuration adjustment of root architectures. In Arabidopsis, root tip contact was essential for triggering the inhibitory growth of primary roots subjected to high NH_4^+ treatments [13]. Whereas leaf contact and absorption of toxic NH_4^+ obstructed primary polar transportation of IAA to the roots through the pathway of AUX1 thereby inhibited the emergence of lateral roots [14]. In rice, high concentrations of NH_4^+ supplied to roots strongly inhibited seminal root elongation and led to reduction of plant growth [15–19]. Mechanisms of plant responses to external NH_4^+ stresses had been summarized as the accumulative consequences of ion imbalance, intracellular pH disturbance, carbon limitation, charge/hormone imbalance and oxidative stresses [20–24]. The work on Arabidopsis *hsn/vtc* mutants provided further molecular evidence that GDP-mannose pyrophosphorylases were involved in protein N-glycosylation modulated inhibition of root elongation under NH_4^+ stresses [25–27]. Moreover, phytohormone signals were reported to interact with NH_4^+ supply thus to regulate plant metabolism, growth and development [13, 14, 24, 25, 27, 28]. And these responsive components are closely related to high external NH_4^+ supply but seemingly independent of internal NH_4^+ accumulation.

Due to the long-time cultivation in paddy soils wherein NH_4^+ as the main N source, rice has been hailed as an uncommon NH_4^+ -tolerant species. Researches on (molecular) mechanism of rice response to excessive NH_4^+ are ongoing. Under condition of high NH_4^+ supply, GABA alleviates NH_4^+ toxicity through reducing NH_4^+ accumulation and assimilation capacity for a energy saving [29]; OsPTR6 could promote rice root growth by enhancing *OsAMT1* expression and GS activity but at the expense of decreasing NUE[30]; OsSE5 that encodes the heme-heme oxygenase 1 dedicates to relieve NH_4^+ toxicity by reinforcing antioxidant defense system [18]. The transcriptome analysis illustrates that the phenylpropanoid and amino acid metabolism show importance in shoot while defense and signaling pathways play the leading role in rice root when stressed by excessive NH_4^+ [19].

In general, the current recognitions on plant responses to NH_4^+ stresses mainly emphasized their physiological and molecular mechanisms in remodeling the (root) architectures that depended on the occurrence of measurable (root) phenotypes accumulated within a relatively longer time course of exposure to NH_4^+ stresses. To this respect, 'mixed' influences between specific NH_4^+ stress responses and endogenous changes along the course of plant growth and development seemed inevitable. Therefore, to

one hand, the nature of high NH_4^+ stresses and the primary responses remained to be specifically clarified; to the other hand, the actions of internal NH_4^+ excess resulted from over-dosed application of N fertilizers needed to be addressed in rice plant without or beyond the frame of a root phenotype.

A previous study implied that the adjustment of carbohydrate metabolisms could be a notable feature in responding NH_4^+ status in rice in a short time period [31]. Environmental stress stimuli such as salinity, drought *etc.* could induce overproduction of reactive oxygenspecies (ROS) and promptly trigger oxidative defense responses [32–33]. Meanwhile, the reduced photosynthetic CO_2 fixation efficiency and alteration of carbohydrate metabolism were speculatively major causes leading to compromised carbon gain and growth retardance [32, 34].

Based on the above descriptions, the present work aimed at revealing the specific nature and mode-of-action of internal NH_4^+ excess stresses in rice plant which represented a practical problem of high-yielding rice farming. Our physiological assessments, gene expression and enzyme activity analyses demonstrated that internal NH_4^+ excess triggered ROS-induced reactions and activated GST-mediated GSH cycles for active radical scavenging that consumed carbohydrates for energization and resulted in carbon scarcity in rice plants. A sucrose feeding treatments effectively alleviated NH_4^+ excess and ROS-induced frustrations supporting that the carbon scarcity was the major constraint of rice plant in dealing with NH_4^+ excess.

Results

Growth inhibition under over-dosed NH_4^+ correlates to an NH_4^+ excess induced ROS burst in rice seedlings

Under persistent treatment with high NH_4^+ (20 mM) for 14d, a significant growth inhibition was observed compared to the control condition (1 mM NH_4^+)(Fig. 1a). The inhibition was more profound in roots showing a biomass reduction of up to 67% (Fig. 1a)and the root/shoot ratio was significantly lowered from approximately 0.5 down to 0.2 (Fig. 1b). Meanwhile, 7 and 5 folds higher concentrations of free NH_4^+ were measured in roots and shoots, respectively (Fig. 1c).The inhibitory effect by high NH_4^+ subjected to plant roots was however, a well known phenomenon as described by numerous reports elsewhere (see Introduction). Efforts have be extensively made on the elucidation of molecular mechanisms involved in root architecture adjustments in response to the accumulation of relatively long-term (several days or longer) stress effects impended by high NH_4^+ treatments. Here to reveal early responsive reactions that could be the trigger of the accumulative responses (growth modifications), a prompt status of internal NH_4^+ excess is necessarily to be established without causing visible changes in plant growth (especially roots). Therefore, L-methionine-D,L-sulfoximine (MSX), a potent inhibitor of the primary NH_4^+ assimilation pathway mediated by the activity of glutamine synthetases [35] was applied (1

mM) for 4 h in the presence of high NH_4^+ (20 mM). This treatment resulted in similar increases in free NH_4^+ and rapidly achieved 'saturable' NH_4^+ excess in both roots and shoots (Fig. 1d), providing a facilitated approach for subsequent identification of genes or processes involved in this response. In line with the accumulation of free NH_4^+ , bursts of reactive oxygen species (ROS) were observed (Fig. 1e), implying possible occurrence of oxidative injuries or ROS-induced reactions triggered by internal NH_4^+ excess.

RNA-Seq analysis for preliminary identification of genes modulated by NH_4^+ excess

According to above description, rice seedlings were treated with high NH_4^+ in the presence of 1mM MSX for 4 h to establish an internal environment of NH_4^+ excess. Then RNA-Seq analyses were carried out to seek for molecular responses related to this circumstance. Respectively 1077 and 1040 differentially expressed genes (DEGs) were obtained from roots and shoots, with > 2 fold changes in their transcriptional levels (Additional file1). Based on the GO classification, these genes mainly belonged to "metabolic process", "molecular function", "binding" and "biological process" (Additional file 2). Further KEGG pathway analysis revealed possible involvements of the responsive genes (DEGs) in stress response, photosynthetic adjustment, carbohydrate and amino acid metabolisms, preparation of hormone signaling pathways and re-adjustment of NH_4^+ transport (Additional file 3). The significantly regulated genes were further summarized below within the framework of major processes they participate.

Activation of GSH cycle for ROS scavenging

Following the acute plant NH_4^+ excess and the bursts of ROS (Fig. 1c, d, e), a most remarkable response was the strong induction of glutathione S-transferases (GST) genes (Fig. 2). Eleven GST genes were typically upregulated for >7 or even some tens to hundreds fold both in roots and shoots (Fig. 2a, b, genes#1–11). Among those GSTs, a OsGSTU4 (Os10g0528300, Fig. 2a, gene#11) was the most severely induced by >300 and >600 fold in roots and shoots respectively, followed by 2 putative GST genes (Os10g0481300 and Os10g0527800) that were upregulated by 50–100 fold in both parts. Whereas Os10g0525500 (77 fold) and Os03g0785900 (90 fold) showed strong induction in roots and shoots respectively (Fig. 2a, b). Since GSTs catalyze the transfer of superoxide free radicals to reductive glutathione (GSH) that leads to the detoxification of the oxidants, these changes in GST gene expression provide indications for the critical involvement of the GSH cycle in scavenging the NH_4^+ excess induced ROS.

The enhanced GST activity accelerates the consumption and conversion of GSH to its oxidized form (GSSG). In line with strengthened demand of reducing power, a putative glutathione reductase gene

(Os10g0415300) responsible for the recruitment of GSH was moderately upregulated (~ 8 fold) in roots and vigorously enhanced by 70 fold in shoots (Fig. 2a). Meanwhile, a NADH dehydrogenase gene (Os07g0564500) was stimulated by 127 folds in shoots, partly reflecting the coupling of energization and reducing power with the operation of the GSH cycle (Fig. 2a).

In addition to profound changes related to the GSH cycle, 7 peroxidase genes were suppressed in roots whereas a putative 1-Cys peroxiredoxin B gene (Os07g0638400) was significantly induced in both roots (19 fold) and shoots (179 fold) (Fig. 2a), corresponding to the contradictory roles of peroxidases in the cleavage / homeostasis maintenance of ROS [36].

Suppression of photosynthesis components and contrasting regulation of energy producing carbohydrate metabolism

The chlorophyll a/b binding proteins of light-harvesting complexes (LHCs), also known as antenna proteins, are involved in gathering light energy (photons) of the primary reaction of photosynthesis [37]. Then trapped photons and electrons are transported to reaction center for further photochemical reactions. Disruption of these processes by photodamage, herbicides, or accumulation of highly active radicals will obviously hinder the progress of photosynthesis. Upon a prompt (4 h) NH_4^+ excess treatment, 6 genes coding for the LHC antenna proteins (4 LHC II and 2 LHC I, respectively), a PS I and a PS II reaction center genes were almost evenly suppressed by approximately 5 fold (Fig. 3), indicating the onset of the reduction of efficiencies of photon gathering and transfer. It would be easily supposed that apparent suppression of photosynthesis would accumulate along the progress of NH_4^+ excess stress and growth inhibition would consequently occur. Meanwhile, Os12G0292400 coding for the small chain of Rubisco, the key enzyme catalyzes the fixation / assimilation of CO_2 , was downregulated by ~ 5 fold (Fig. 3), providing further indication of compromised photosynthetic carbon production. Therefore, plant NH_4^+ excess initiates and probably also develops the disruption of photosynthesis by interfering in the primary reaction and the Calvin Cycle.

Radical scavenging enzymes are activated and energized by the ATP producing processes including glycolysis and the TCA pathways. However, several genes involved in glycolysis and the TCA cycle were contrastingly regulated in roots and shoots (Fig. 4). In roots, genes coding for 2,3-bisphosphoglycerate-independent phosphoglycerate mutase (Os05g0482700, gene#33) and fructose-bisphosphate aldolases (Os08g0120600, gene#34 and Os01g0905800, gene#35) of glycolysis, isocitrate dehydrogenase (Os05g0573200, gene#36) and malate dehydrogenase (Os05g0574400, gene#37) of the TCA cycle were down-regulated by 6–10 fold following 4h of NH_4^+ excess treatments (Fig. 4a).

Meanwhile genes involved in glycogen breakdown were suppressed in roots (Fig. 4a): phosphoenolpyruvate carboxykinase (Os10g0204400, gene#32, –19 fold), beta-glucosidase (Os09g0491100, gene# 40, –11 fold), beta-glucosidase (Os02g0131400, foldgene#41, –15 fold), beta-D-xylosidase 4 (Os04g0640700, gene#42, –7 fold), sucrose synthase (Os03g0401300, gene#43, –8 fold), beta-fructofuranosidase (Os02g0106100, gene#44, –11 fold). To the contrary, enhanced glycolysis /

glycogen breakdown in shoots could be indicated by the upregulation of related genes (Fig. 4b): glucose-6-phosphate 1-dehydrogenase (Os02g0600400, gene#39, +5 fold), inorganic pyrophosphatase (Os05g0438500, gene#49, +18 fold), phosphoenolpyruvate carboxykinase (Os10g0204400, gene#32, +34 fold), beta-glucosidase (Os05g0366600, gene#47, +12 fold), beta-glucosidase (Os09g0511600, gene#48, +20 fold). Notably, a pyruvate decarboxylase gene (Os05g0469600, gene #38) of glycolysis, was specifically induced in shoots (Fig. 4b). In addition, two genes Os06g0222100 and Os08g0445700 coding for trehalose 6-phosphate synthase/phosphatases were induced by respectively 15 and 13 fold in roots (Fig. 4a, genes #45, 46), suggesting enhanced biosynthesis of the 'survival substance' [32] trehalose induced by NH_4^+ excess stress.

Sucrose feeding alleviates NH_4^+ excess stress responses

The above analyses revealed rather frustrating responses to NH_4^+ excess stress in rice plant that closely associated with the consumption of carbohydrates for energy demand. Hence a sugar scarcity could accumulatively (to a longer time course) result in growth inhibition. To test this hypothesis, we fed 1% of sucrose as a sugar compensation to the high NH_4^+ (20 mM) hydroponics for 24 h. This treatment compensated the sucrose consumption at high NH_4^+ and allowed the sucrose contents in roots and shoots to restore to equivalent levels of the control (1 mM NH_4^+) conditions (Fig. 5a). The sucrose feeding treatments further increased the free NH_4^+ contents in roots, but significantly reduced NH_4^+ accumulation to the shoots (Fig. 5b).

As the *AMT1* (*OsAMT1;1*(Os04g0509600/LOC_Os04g43070), *OsAMT1;2*(Os02G0620500/LOC_Os02g40710) and *OsAMT1;3*(Os02G0620600/LOC_Os02g40730)) expression level that previously suppressed by high NH_4^+ in roots (respectively by 3, 67 and 6 fold) subsequently restored to close to their initial levels (at 1 mM NH_4^+) with the supplement of sucrose to the high NH_4^+ hydroponics (Fig. 5c). This implied a release of ammonium transporting activity facilitated by the supply of sucrose, thus to contribute to enhanced NH_4^+ accumulation in roots under high NH_4^+ plus sucrose condition, whereas the reduced NH_4^+ content under the same condition in shoots indicated probably the efficient utilization of NH_4^+ upon the addition of sucrose (Fig. 5b). Meanwhile the GS (Fig. 5d) and GOGAT (Fig. 5e) activities were respectively enhanced by 17 % (GS) and 29% (GOGAT) in roots following the sucrose feeding treatments, indicating a restoration of NH_4^+ assimilation activities from initial suppression by NH_4^+ excess.

Upon the compensation of sucrose source, the total ROS contents in both roots and shoots were lowered down by 20–30%, close to the levels determined at control (1 mM NH_4^+) conditions (Fig. 6a). Accordingly, the GSH content and GST activity were significantly reduced to the initial levels (at 1 mM NH_4^+), no longer showing strong induction by NH_4^+ excess (Fig. 6b, c). Unexpectedly, no significant changes were

observed with the activities of classical defense enzymes CAT, POD and SOD under either treatment (Fig. 6d, e, f). Together with the gene expression analyses (Fig. 2), our results demonstrated that the activation of GSH reducing pathway is probably a featured response of rice in dealing with NH_4^+ excess and ROS accumulation. Finally, in consistent with the decreased level of ROS, Rubisco activity was elevated by 24% (compared with high NH_4^+) in shoots with the presence of sucrose feeding (Fig. 6g), suggesting enhanced efficiency of primary CO_2 fixation activity.

Taken together, this set of experiments indicated that sucrose feeding could effectively alleviate rice plant from carbon scarcities exerted by internal NH_4^+ excess and ROS stresses.

Discussion

Due to particular water-flooding and anaerobic growth environment, NH_4^+ is the major form of N nutrient available to paddy rice. Current farming practice of the overuse of N fertilizers to satisfy the N demand of high-yielding rice creates a persistent circumstance of NH_4^+ excess around the roots and inside the plant for rice to cope with [10–12, 38, 39]. Therefore, a study focuses on such farming-intervened special circumstances would be helpful in discovering 'bottlenecked' constraints and adaptation strategies related to the (molecular) physiological and agronomic respects of N performances in rice.

NH_4^+ stresses and toxicities have been considered as a major human-intervened environmental distress exerted on plants and attracted extensive research interests. Researches on these topics have focused on the identification of mechanisms or pathways that primarily modulate the biological modifications of root architectures [20–24]. Solid evidences have shown the re-configuration of plant root morphology in response to NH_4^+ stresses is tightly controlled through the interactions with plant hormone signaling pathways [13, 14, 24, 25, 27]. Whereas NH_4^+ toxicities could be attributed to ion imbalances [20], intracellular pH disturbance [40], energy consumption due to invalid NH_4^+ cycles in roots [13, 41].

Assessments of NH_4^+ stress responses in plant roots and its biological toxicities, to a great extent, rely on the establishment of measurable growth phenotypes that requires effects or reactions to accumulate for a desired time course. These analyses are obviously important in addressing the mode-of-action of physiological effects or processes developed along the progresses of the treatments. To the other hand, since plants grow and develop during the experimental periods, these accumulative observations might be not satisfactory for capturing the initial reactions or the nature of NH_4^+ excess stresses. Therefore, in the present work, we established prompt methods for identification of the early responses especially to internal NH_4^+ excess without causing visible growth changes by blocking the primary NH_4^+ assimilation with MSX.

Our results demonstrate that internal NH_4^+ excess (accumulation of free NH_4^+) correlates to a burst of ROS radicals (Fig. 1c, d, e) and activates (may be specifically) the GST-mediated GSH cycling rather than classical antioxidant defense enzymes (such as CAT, POD and SOD) for ROS scavenging (Fig. 2, 6).

Therefore, our results support the notion that the initial nature of NH_4^+ excess in rice plant is probably the induction of ROS bursts that triggers the downstream reactions.

Following the ROS radical bursts, genes involved in photosynthetic photon capture and the most important enzymes for primary CO_2 assimilation-Rubisco (gene expression and enzyme activity as well) were suppressed (Fig. 3, 6g), indicating the carbon income is reduced from its origin of production. Next, to support the sharp energy demand of ROS cleavage through the GSH cycling, we observe strong inductions especially in shoots, of genes involved in ATP-generating reactions (glycolysis and glycogen breakdown, Fig. 4) and enhanced gene expressions related to processes of [gluconeogenesis](#) and structural sugar breakdown to fill up the consumption of 'active' carbons (Fig. 4). To this end, the carbon consumption and thereby the carbon scarcity associated with NH_4^+ excess in rice plant resemble the metabolism alterations caused by well known abiotic stresses, such as drought or salinity stresses described in other plant species [32, 34].

The oxidative inhibition of photosynthesis and the scarcity in active carbon source initiated by internal NH_4^+ excess are further supported by the experiments of sucrose feeding (Fig. 5, 6). Sucrose is the major form of active carbon source in the phloem and can be transported and allocated in plant tissues and organs [42]. Therefore we choose sucrose for carbon compensation experiments. Efficient NH_4^+ uptake through AMT transporters is closely related to the removal of the substrate by GS-mediated assimilation processes, ineffective removal or accumulation of NH_4^+ would hinder the uptake of the ions—a phenomenon of so-called feedback inhibition [43–45]. The strong suppression of root-specific AMT genes, *OsAMT1;2* and *OsAMT1;3* under rapid NH_4^+ excess conditions (Fig. 5c) and reduction of GS and GOGAT activities (Fig. 5d, e) provide further evidence of such feedback regulation in rice. Here, upon the carbon compensation by sucrose feeding, the NH_4^+ assimilation activities restore to normal rates (normal NH_4^+ , 1 mM) and the AMT expression levels are accordingly enhanced (Fig. 5c, d, e), supportedly suggesting that carbon scarcity may be the major cause that leads to feedback inhibition of NH_4^+ uptake. This speculation could find further supports by the reduction of free NH_4^+ and ROS contents to the normal levels as observed in the control (1 mM NH_4^+) conditions upon a 24 h of sucrose feeding (Fig. 5b, 6a). With the [tranquillness](#) of ROS burst, enhanced GSH content and GST activity are no longer essential (Fig. 6b, c) and the Rubisco CO_2 fixation enzymes restore to act at normal (or even facilitated) activity (Fig. 6g). Thus, the NH_4^+ excess stress caused growth inhibition as evidenced elsewhere could be interpreted by the carbon scarcity resulted from the reduction of carbon synthesis and violent carbon consumption for energy production.

Conclusions

The present work reveals that the essential nature of internal NH_4^+ excess stresses in rice plant is closely correlated to its accompanying ROS bursts. Coping with ROS bursts by energy and carbon consumption processes leads to carbon scarcity that accumulatively inhibits plant growth. Sucrose feeding alleviates

stress responses, indicating that enhancement of carbon income processes could be a useful approach for further improving nitrogen performances of rice under current farming practice of N fertilizer overuse.

Methods

Plant growth and treatments

Rice seeds of *Oryza. sativa* ssp. Japonica *Nipponbare* were obtained from Prof. Yingguo Zhu's group, College of Life Sciences, Wuhan University. The seeds were sterilized, germinated and seedlings were grown in a growth chamber according to previously described [31]. The growth chamber was set with 16/8 h day/night, 27/25°C, day/night; light intensity was $400 \mu\text{mol m}^{-2} \text{s}^{-1}$, relative humidity was set at 70%. Seedlings were grown in the IRRI solution [31]. The pH of hydroponics was buffered to 5.7 with 10 mM MES and renewed every 48h. For long-term growth tests, uniform seedlings of 7 d were treated with either 1 mM (control) or 20 mM (high NH_4^+) NH_4Cl supplemented to nitrogen-free IRRI solutions for further 14 days with daily refreshment of the culture solutions. To achieve a rapid NH_4^+ excess in rice plants without causing a growth discrepancy, so that NH_4^+ excess-responsive genes could be analyzed at the early stages of responses, 10-d old seedlings were promptly treated with 'control' or 'high NH_4^+ ' (see above) for 4 h in the presence of 1 mM methionine sulfoximine (MSX, a potent inhibitor of glutamine synthetase) to block the assimilation of NH_4^+ .

For carbon source feeding experiments, 14-d old seedlings were treated with control (1 mM NH_4^+) or high NH_4^+ (20 mM NH_4^+) in IRRI solution in the presence of 1% (w/v) sucrose for 24 h. To avoid the burst of microbes associated with sucrose-containing hydroponics, antibiotics penicillin (50 mg L^{-1}) and chloramphenicol (25 mg L^{-1}) were included to the culturing solution according to Lejay's description [46]. Also in order to prevent possibly undesired impacts, the treatment was limited to within 24 h.

RNA-Seq and quantitative real-time PCR analyses

Total RNAs from treated root or shoot samples was extracted with TRIzol total RNA extraction kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. For RNA-Seq analysis, RNAs from control (1mM NH_4^+ or high NH_4^+ plus 1 mM MSX treated (4 h) tissue samples were used for library construction and sequencing. Data extraction, identification of differentially expressed genes (DEGs) and functional annotation were analyzed according to our previous work [31]. DEGs were designated with expression fold changes greater than 2 ($p < 0.05$) between the rapid NH_4^+ accumulation (high NH_4^+ + MSX) and the control conditions.

Quantitative real-time PCR (qRT-PCR) analyses was carried out to reveal possible responses at the gene expression level related to special conditions such as NH_4^+ excess stress or sucrose feeding treatments. About 1 μg of total RNA was used to synthesize first-strand cDNA using the PrimeScript™ RT Master Mix

(Perfect Real Time, TaKaRa, Japan) according to the manufacturer's description. Primer sequences used for qRT-PCR were listed in Additional file4. Thermocycling and fluorescence detection were performed with C1000 Thermal Cycler CFX96 Real-Time System (Bio-Rad) using the SYBR Premix Ex Taq (TaKaRa, Japan) as indicated by the manufacturer's protocol. The reaction was performed under the following conditions: 95 °C for 30 s, followed by 44 cycles of 95 °C for 10 s, 60 °C for 15 s and 72 °C for 15 s. For fold change analysis, gene expression abundance was quantified with $-2^{\Delta\Delta Ct}$ and normalized against the internal OsActin gene. PCR amplifications were repeated three times using cDNA templates synthesized from three independent plant samples.

Tissue free NH_4^+ , GSH and sucrose content assays

Fresh root or shoot samples (0.2 g) were ground into fine powder in liquid N_2 and homogenized in 5 ml of 0.3 mM sulfuric acid. The supernatant was harvested by centrifugation with 20000 g at 4°C for 20 min. Aliquots of supernatant (200 μL) were mixed with 4.9 mL each of phenol-sodium nitroprusside solution and alkaline hydrochlorite solution according to the method of Weatherbur [47]. The color reaction was allowed to develop at room temperature for 1h before the colorimetric absorbance been measured at 625 nm. The tissue GSH content was measured according to Cheng's method [48]. The tissue sucrose extraction was carried out according to Sonnewald's method [49], and the tissue sucrose contents determination was measured based on Stitt's description [49, 50].

Determination of total ROS and ROS scavenging enzyme activities

Total reactive oxygen species (ROS) contents induced by internal NH_4^+ excess were assessed by 2',7'-Dichlorofluoresceindiacetate ($\text{H}_2\text{DCF-DA}$) method [51]. Fresh root or shoot samples were first made into single cell suspensions with single cell suspension medimachine. Then $\text{H}_2\text{DCF-DA}$ was added to 200 μL single cell suspensions to a final concentration of 10 μM , mixed and incubated at 37°C for 30 min. Cells were pelleted by centrifugation at 1000 g for 10min, washed twice with PBS, and diluted with PBS for fluorescence assay. The absorbance was determined on a fluorescence microplate reader (BioTek Instruments, Winooski, VT) at an excitation wavelength of 500 nm and an emission wavelength of 530 nm according to the descriptions of Karlsson and Sun [52, 53].

For antioxidative enzyme activity analyses, 0.2 g of fresh root or shoot samples were ground in liquid N_2 , homogenized and crude extracts were used for the measurements of CAT, POD and SOD activities as previously described [32]. The specific activity of GST was assayed in the supernatant by following the increase of absorbance at 340 nm using GST Assay Kit according to the manufacturer instructions (CS0410, Sigma, USA). One unit of activity was defined as the amount of enzyme required to form 1 μM product per minute at 30°C. Enzyme activities were expressed as $\text{U. mg}^{-1} \text{FW}$.

Measurement of GS, GOGAT and Rubisco activities

To prepare the crude enzyme extracts, roots or shoots of each sample were ground into fine powder with liquid N₂ and homogenized with 50 mM Tris–HCl buffer (pH 7.6, containing 10 mM MgCl₂, 1mM EDTA, 1mM β-mercaptoethanol and 4% (w/v) polyvinylpolypyrrolidone–40) using a chilled pestle and mortar. The homogenate was centrifuged at 15000 g for 30 min at 4°C and the supernatants were used for the determination of enzyme activities. The glutamine synthetase (GS) activity was measured according to Sakurai's description [54]. One unit of GS activity was expressed as the amount of enzyme catalyzing the formation of 1 μmol γ-glutamyl hydroxamate per min at 37°C [55]. The glutamate synthase (GOGAT) activity in the supernatants was determined by the conversion of 2-ketoglutarate to glutamate in a reaction mixture containing 200 mM KH₂PO₄-KOH pH 7.5, 10 mM glutamine (Gln), 10 mM 2-ketoglutarate, 0.14 mM NADH [56]. One unit of GOGAT activity was defined as the oxidation rate of 1 nmol NADH per min at 30°C. And the Rubisco activity was measured according to [57]. One unit of Rubisco activity was defined as the oxidation rate of 1 nmol NADH per min at 25°C.

Statistical analysis

Experiment data were expressed as means ± S. E. M. of 3 independent replicates. Statistical differences were evaluated by Duncan's or t-test with SPSS 13.0 and the level of statistically significant difference was set at p<0.05.

Declarations

Supplementary information

Additional files

Additional file 1: Table S1. Summary of total DEGs identified in rice roots and shoots following a 4 h rapid NH₄⁺ accumulation treatment.

Additional file 2: Table S2. GO enrichment analysis of DEGs in rice roots and shoots following a 4 h rapid NH₄⁺ accumulation treatment.

Additional file 3: Table S3. KEGG Enrichment Analysis of DEGs in rice roots and shoots following a 4 h rapid NH₄⁺ accumulation treatment.

Additional file 4: Table S4. The sequences of primers used for real-time RT-PCR in this research.

Abbreviations

AMT: ammonium transporter; CAT: catalase; CO₂: carbon dioxide; DEGs: Differentially Expressed Genes; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; GOGAT: glutamate synthase; GS:

Glutamine synthetase; GSH: Glutathione; GST: Glutathione S-transferase;

H₂DCF-DA: 2',7'-Dichlorofluoresceindiacetate; LHCs: light-harvesting complexes; N, nitrogen; NH₄⁺: ammonium; qRT-PCR: Quantitative Reverse Transcription Polymerase Chain Reaction; POD: peroxidase; ROS: Reactive Oxygen Species; RNA-Seq: RNA sequencing; SOD: superoxiddismutase; Rubisco: Ribulose-1, 5-bisphosphate carboxylase/oxygenase; TCA cycle; Tricarboxylic acid cycle

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data supporting the conclusions of this article are provided within the article (and its additional files).

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

YS and SY conceived and designed the research. SY, DH, MJ prepared samples for RNA-Seq analysis, performed quantitative PCR validation and analyzed data. SY, DH, MJ, YL, ZL, YH and TC carried out physiological/biochemical assays and data analyses. SY and YS wrote the manuscript. All authors read and approved the manuscript.

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Figures

Figure 1

Biological and physiological analyses of NH_4^+ excess responses of rice. Rice seedlings aged 7d were subjected to NH_4^+ treatments for 14d (a-c, e). (a) Fresh biomasses of roots and shoots. (b) Root-shoot ratios. (c) Free NH_4^+ content and (e) total ROS content in response to NH_4^+ treatments. (d) Prompt NH_4^+ excess simulation by treating with high NH_4^+ for 4h in the presence of 1 mM MSX. Rice seedlings used

for this experiment were 10d old. Values indicated means $\pm$ SE of three independent experiments. ** and *** represented statistical significances at $p \leq 0.01$ and 0.001 , respectively.

Figure 2

Gene expression analyses of responsive ROS scavenging genes. (a) Differentially expressed genes obtained by RNA-Seq were illustrated in relation to the major pathways they were involved. Colored columns corresponded to fold changes of the transcripts as indicated by the definition of color gradients (bottom). The symbol ‘+’ and the red gradient columns represented up-regulated genes and the fold of induction; while ‘-’ and the green gradient columns referred to down-regulated genes. (b) The qRT-PCR validation of randomly selected genes coding for ROS scavenging systems. The relative expression levels were normalized against OsActin . Values indicated were means of three different replicates. Numbered responsive genes were annotated as followings: 1 (Os01g0949700), putative glutathione S-transferase; 2 (Os03g0785900), probable glutathione S-transferase GSTU1; 3 (Os01g0369700), putative glutathione transferase 4; 4 (Os01g0949800), putative glutathione S-transferase; 5 (Os01g0949750), putative glutathione S-transferase; 6 (Os10g0365200), glutathione S-transferase; 7 (Os10g0527800), glutathione S-transferase OsGSTU12; 8 (Os10g0481300), glutathione S-transferase; 9 (Os10g0525500), glutathione S-transferase parC; 10 (Os01g0372400), putative glutathione S-transferase; 11 (Os10g0528300), glutathione S-transferase OsGSTU4; 12 (Os10g0415300), glutathione reductase; 13 (Os08g0557600), monodehydroascorbate reductase; 14 (Os07g0638400), 1-Cys peroxiredoxin B; 15 (Os05g0499300), peroxidase 1; 16 (Os07g0677300), peroxidase 2; 17 (Os05g0134800), Class III peroxidase 67; 18 (Os02g0236600), Class III peroxidase 27; 19 (Os03g0234900), Class III peroxidase 39;

20 (Os03g0368000), Class III peroxidase 42; 21 (Os06g0695300), Class III peroxidase 92; 22 (Os07g0564500), NADH dehydrogenase [EC:1.6.99.3].

Figure 3

Responsive genes involved in photosynthesis. Differentially expressed genes obtained by RNA-Seq were illustrated in relation to the major processes they were involved. Colored columns corresponded to fold changes of the transcripts as indicated by the definition of color gradients (bottom). The symbol '+' and the red gradient columns represented up-regulated genes and the fold of induction; while '-' and the green gradient columns referred to down-regulated genes. Numbered responsive genes were annotated as followings: 23 (Os03g0592500), light-harvesting complex II chlorophyll a/b binding protein 2 (LHCB2); 24 (Os07g0558400), light-harvesting complex II chlorophyll a/b binding protein 4 (LHCB4); 25 (Os01g0720500), light-harvesting complex II chlorophyll a/b binding protein 1 (LHCB1); 26 (Os09g0346500), light-harvesting complex II chlorophyll a/b binding protein 1 (LHCB1); 27 (Os03g0333400), photosystem II Psb27 protein (psb27); 28 (Os08g0560900), photosystem I subunit II (psaD); 29 (Os06g0320500), light-harvesting complex I chlorophyll a/b binding protein 1 (LHCA1); 30 (Os02g0197600), light-harvesting complex I chlorophyll a/b binding protein 3 (LHCA3); 31 (Os12g0292400), ribulose-bisphosphate carboxylase small chain [EC:4.1.1.39] (rbcS).

Figure 4

Responsive genes involved in carbohydrate metabolisms in roots (a) and shoots (b). Differentially expressed genes obtained by RNA-Seq were illustrated in relation to the major processes they were involved. Colored columns corresponded to fold changes of the transcripts as indicated by the definition of color gradients (bottom). The symbol '+' and the red gradient columns represented up-regulated genes and the fold of induction; while '-' and the green gradient columns referred to down-regulated genes. Numbered responsive genes were annotated as followings: 32 (Os10g0204400), phosphoenolpyruvate carboxykinase (ATP) [EC:4.1.1.49](pckA); 33 (Os05g0482700), 2,3-bisphosphoglycerate-independent phosphoglycerate mutase [EC:5.4.2.12](gpmI); 34 (Os08g0120600), fructose-bisphosphate aldolase, class I [EC:4.1.2.13](ALDO); 35 (Os01g0905800), fructose-bisphosphate aldolase, class I [EC:4.1.2.13](ALDO); 36 (Os05g0573200), isocitrate dehydrogenase [EC:1.1.1.42](IDH); 37 (Os05g0574400), malate dehydrogenase [EC:1.1.1.37](MDH2); 38 (Os05g0469600), pyruvate decarboxylase [EC:4.1.1.1]; 39 (Os02g0600400), glucose-6-phosphate 1-dehydrogenase [EC:1.1.1.49](G6PD); 40 (Os09g0491100), beta-glucosidase [EC:3.2.1.21]; 41 (Os02g0131400), beta-glucosidase [EC:3.2.1.21]; 42 (Os04g0640700), beta-D-xylosidase 4 [EC:3.2.1.37](XYL4); 43 (Os03g0401300), sucrose synthase [EC:2.4.1.13]; 44 (Os02g0106100), beta-fructofuranosidase [EC:3.2.1.26](sacA); 45 (Os08g0445700), trehalose 6-phosphate synthase/phosphatase [EC:2.4.1.15 3.1.3.12](TPS); 46 (Os06g0222100), trehalose 6-phosphate phosphatase [EC:3.1.3.12](otsB); 47 (Os05g0366600), beta-glucosidase [EC:3.2.1.21]; 48 (Os09g0511600), beta-glucosidase [EC:3.2.1.21]; 49 (Os05g0438500), inorganic pyrophosphatase [EC:3.6.1.1].

Fig. 5

Figure 5

Effects of sucrose feeding on NH₄⁺ accumulation, uptake and assimilation processes. Seedlings aged 10 d were subjected to control (1mM NH₄⁺), high NH₄⁺ (20 mM) or high NH₄⁺ + suc (20 mM NH₄⁺ + 1% sucrose) treatments for 24h. (a) Sucrose content, (b) free NH₄⁺ content, (c) the expression profiles of OsAMT1;1, OsAMT1;2 and OsAMT1;3 determined by qRT-PCR, (d) GS enzyme activity, (e) GOGAT enzyme activity. Data were means ± SE of three biological replicates. Letters on the bars represented statistical significances.

Figure 6

Effects of sucrose feeding on ROS accumulation, ROS scavenging enzymes and Rubisco activities. Seedlings aged 14 d were subjected to control (1mM NH₄⁺), high NH₄⁺ (20 mM) or high NH₄⁺⁺ suc (20 mM NH₄⁺ + 1% sucrose) treatments for 24h. (a) Total ROS accumulation represented by fluorescence degrees, (b) GSH content, (c-g) Enzymes activity assays for (c) GST, (d) CAT, (e) POD, (f) SOD and (g)

Rubisco. Experimental conditions were the same as described in Figure 5. Data were expressed as means $\pm$ SE of three biological replicates. Letters on the bars represented statistical significances.

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