Ammonium Uptake and Assimilation are Required for Rice Defense against Sheath Blight Disease

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Nitrogen (N) is an important nutrient for plant growth and yield production, and rice grown in paddy soil mainly uses ammonium (NH_4^+) as its N source. Previous studies have shown that N status is tightly connected to plant defense; however, the roles of NH_4^+ uptake and assimilation in rice sheath blight disease response have not been studied previously. Here, we analyzed the effects of different N sources on plant defense against *Rhizoctonia solani*. The results indicated that rice plants grown in N-free conditions had higher resistance to sheath blight than those grown under N conditions. In greater detail, rice plants cultured with glutamine as the sole N source were more susceptible to sheath blight disease compared to the groups using NH_4^+ and nitrate (NO_3^-) as sole N sources. N deficiency severely inhibited plant growth; therefore, *ammonium transporter 1;2* overexpressors (*AMT1;2 OXs*) were generated to test their growth and defense ability under low N conditions. *AMT1;2 OXs* increased N use efficiency and exhibited less susceptible symptoms to *R. solani* and highly induced the expression of *PBZ1* compared to the wild-type controls upon infection of *R. solani*. Furthermore, the *glutamine synthetase 1;1* (*GS1;1*) mutant (*gs1;1*) was more susceptible to *R. solani* infection than the wild-type control, and the genetic combination of *AMT1;2 OX* and *gs1;1* revealed that *AMT1;2 OX* was less susceptible to *R. solani* and required *GS1;1* activity. In addition, cellular NH_4^+ content was higher in *AMT1;2 OX* and *gs1;1* plants, indicating that NH_4^+ was not directly controlling plant defense. In conclusion, the present study showed that the activation of NH_4^+ uptake and assimilation were required for rice resistance against sheath blight disease.

Keywords: AMT, assimilation, sheath blight, defense, *GS1;1,* rice

Abbreviations: AMT: ammonium transporter; Os: *Oryza sativa*; GS: glutamine synthetase; N: nitrogen; *R. solani*: *Rhizoctonia solani;* OX: overexpression; ORF: open reading frame; *PBZ1:* Probenazole-inducible gene

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Introduction

In most soils, nitrate (NO_3^-) and ammonium (NH_4^+) represent the major forms of nitrogen (N) uptake in higher plants. The NO_3^- and NH_4^+ ions accumulate in cells by direct uptake from the rhizosphere via NO_3^- transporters and NH_4^+ transporters, with NO_3^- further metabolized to NH_4^+. The NH_4^+ is then assimilated into glutamate via the glutamine synthetase (GS)/glutamate synthase cycle. Glutamine and asparagine have been identified as the major forms of organic N in the xylem and are translocated from the roots to the shoots (Fukumorita and Chino 1982). N is important for plant growth and yield production, and which is counted as 2% of plant dry weight.

N status is also closely associated with plant defense mechanisms. In *Arabidopsis*, *ammonium transporter 1;2* overexpressors (*AMT1;1*) alter basal defense, generating resistance against *Pseudomonas syringe* and *Plectosphaerella cucumerina* (Pastor et al. 2014), and the expressions of NH_4^+ transporters and NO_3^- transporters have been found to be altered by both biotic and abiotic stresses (Fagard et al. 2014). In sorghum, the expressions of *SbAMT3;1* and *SbAMT4* were greatly induced locally in roots colonized by arbuscular mycorrhizal fungi (Koegel et al. 2013). Ammonium supply was reported to protect tomatoes against *Ps. syringae* by an increase in cellular reactive oxygen species levels (Fernandez-Crespo et al. 2015). In addition, amino acids play a role in the defense response of plants. In *Arabidopsis*, inoculation with avirulent *Ps. syringae* pv. *tomato* (*Pto*) expressing *avrRpt2* activates transcription of genes involved in amino acid biosynthesis (Scheideler et al. 2002). Metabolic profiling has also shown that inoculation with virulent or avirulent pathogens alters amino acid contents in *Arabidopsis* (Ward et al. 2010). The *lht1* mutant with reduced levels of proline, glutamine, and alanine shows strong resistance against various types of pathogens including bacteria, filamentous fungi, and oomycetes (Liu et al. 2010). In rice, treatment of the roots with glutamate induces systemic resistance to rice blast disease, partially via salicylic acid signaling (Kadotani et al. 2016). In addition, glutamate treatment has been found to induce defense response genes in rice (Kan et al. 2017). These findings suggest that cellular N levels or N signals are closely associated with plant defense.

Sheath blight is one of the three major diseases in rice and is caused by the fungal pathogen *R. solani* (Savary 1995). Sheath blight threatens rice throughout the growth cycle from seedling to heading and causes lesions on leaves, sheaths, and panicles. During the late stage of infection, the whole plant withers and lodges (Savary et al. 1995). Sheath blight can reduce rice yield by 8–50%, depending on disease severity, crop stage at which the infection occurs, and environmental conditions (Savary 2000). However, the effect of N levels on sheath blight disease has not been reported previously. Here, we analyzed the function of different N sources on sheath blight disease. In addition, the growth patterns of *AMT1;2* overexpressing plants were examined under a relatively low N content as well as the response to sheath blight disease. Further genetic analysis was performed using the *GS1;1* mutant (*gs1;1*) and genetic combination between *gs1;1* and *AMT1;2 OX* to test the function of N uptake and assimilation in sheath blight defense in rice. The data presented here provided new insight into the role of N status on plant growth and pathogen resistance in rice plants.

Materials and Methods

Mutant isolation and plant growth

The *gs1;1* mutant (PFG_3A-09512) was obtained from a rice T-DNA database (http://signal.salk.edu/cgi-bin/RiceGE/) (An et al. 2003). The mutant lines and transgenic plants were derived from the Japonica rice cultivar 'Dongjin.' To analyze N effects on plant growth and defense, rice plants were cultured in N-free Murashige and Skoog (MS) medium (Duchefa) containing 10 mM NH_4NO_3 (high N, HN) or 0.1 mM NH_4NO_3 (low N, LN) for 5 days.

Plants expressing vector construction and transformation

To generate *AMT1;2* overexpressing plants, *AMT1;2* ORF sequences were amplified by polymerase chain reaction (PCR) and further cloned into *Hind*III and *BamH*I sites of the PGA1611 binary vector (Dou et al. 2016). The primers used to amplify *AMT1;2* ORF are listed in Table S1*. *PGA1611-AMT1;2* was transformed into rice calli via the *Agrobacterium*-mediated transformation method (Hiei et al. 1994). The LBA4404 *Agrobacterium* strain was used in the experiments.

Determination of NH_4^+ contents

Enzymatic determination of NH_4^+ contents in roots and shoots of 5-day-old seedlings grown in 0.5X MS medium for 5 days was performed using an F-kit (Roche) according to the manufacturer's instructions (Oliveira et al. 2002).

RNA extraction and real-time reverse transcription PCR (qRT-PCR)

Total cellular RNA was isolated from 30 plant tissues with TRIzol (Takara, Dalian, Liaoning, China) and 1 µg of total RNA was subsequently treated with RQ-RNase free DNase (Promega, Madison, WI, USA) to eliminate genomic DNA contamination. For cDNA synthesis, a GoScript Reverse Transcription Kit was used following the manufacturer's instructions (Promega, Madison, WI, USA). Subsequently, qRT-PCR was performed in triplicate using the SYBR Green Mix (Bio-Rad). PCR products were quantified using the Illumina Research Quantity software Illumina Eco 3.0 (Illumina, San Diego, California, USA) and the values were normalized against *Ubiquitin* levels from the same samples to analyze the ratio for each gene. The primers used for the qRT-PCR are listed in Table S1.

Plant inoculation

All plants were grown under greenhouse conditions (temperature, 23–30 °C, humidity 80%, and 12 h light/12 h dark cycle) at Shenyang Agricultural University, China, and propagated by selfing. One-month-old plants were used for inoculation with *R. solani*

*Further details about the Electronic Supplementary Material (ESM) can be found at the end of the article.

AG1-1A. In brief, a 10 cm long piece was cut from the second youngest leaf of the main tiller and placed on a moistened filter paper in a Petri dish (diameter, 36 cm; height, 2.5 cm). At least 10 leaves from each line were used as one replicate, with four replications per line in a completely randomized design. Colonized potato dextrose agar blocks (diameter, 7 mm) were excised using a circular cutter and placed on the abaxial surface of each leaf piece. The leaves were incubated in a chamber with continuous light at 25 °C for 72 h. The filter paper was kept moist with sterile water. After 72 h, the lesion length of each leaf piece was measured and presented as percentage of area covered with lesions in a leaf (Prasad and Eizenga 2008).

Northern blot analysis

Formaldehyde gels (1.3%) were prepared in MOPS ((3-(N-morpholino) propanesulfonic acid)/ethylenediaminetetraacetic acid [EDTA]) buffer (0.5 M MOPS, pH 7.0; 0.01 M Na_2EDTA, pH 7.5) for Northern blot analysis. Twenty micrograms of RNA for each sample were heat-denatured in a formaldehyde/formamide solution. The gels were electrophoresed and further washed with 10X SSC for 1 h before being blotted onto a Hybond N+ membrane (Amersham Pharmacia Biotech, U.K.). Ethidium Bromide (EtBr) staining for rRNA was used as the loading control. The membrane and a 32P-labeled gene-specific probe were hybridized at 65 °C in Church buffer (1% BSA, 200 μM EDTA, 0.5 M sodium phosphate, 7% SDS). The membranes were autoradiographed using Fuji X-ray film. The *AMT1;2* ORF fragment was used as the probe.

Statistical analysis

Statistical analysis was performed using Prism 5 (GraphPad, San Diego, CA, USA). All data were expressed as means ± standard error. One-way analysis of variance was performed, followed by Bonferroni's multiple comparison test, and the differences were considered significant at $P < 0.05$ (Brady et al. 2015).

Results

N nutrition enhanced susceptibility of rice to sheath blight disease

To analyze the relationship between N status and sheath blight disease, rice seedlings were grown in water for 2 weeks before being transferred to different N sources (N-free, NO_3^-, NH_4^+, and glutamine) containing media culture for another week. Then, the 3-week-old plants were inoculated with *R. solani* AG1-1A strain (Fig. 1A). After 4 days of inoculation, symptoms were analyzed in each treatment group. The results indicated that plants grown in N-free conditions had significantly higher resistance to *R. solani* AG1-1A than plants cultured with N sources (Figure 1B, C). In addition, plants cultured with glutamine as the sole N source were more susceptible to sheath blight disease, whereas those cultured with NO_3^- were less susceptible and the NH_4^+ supplemented group exhibited moder-
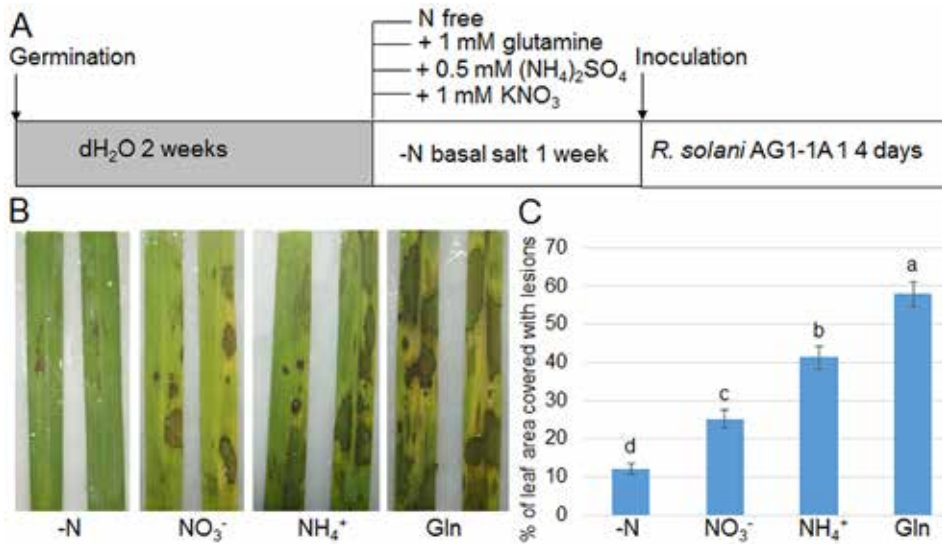
Figure 2. The effects of N concentrations on rice seedling growth. (A) Wild-type plants were grown in the modified MS medium containing 10 mM NH_4NO_3 (high nitrogen, HN) or 0.1 mM NH_4NO_3 (low nitrogen, LN) for 5 days. Scale bar = 1 cm. (B) The seminal shoot and root lengths of plant shown in (A) were calculated (n > 10). Different letters indicate significant differences at P < 0.05
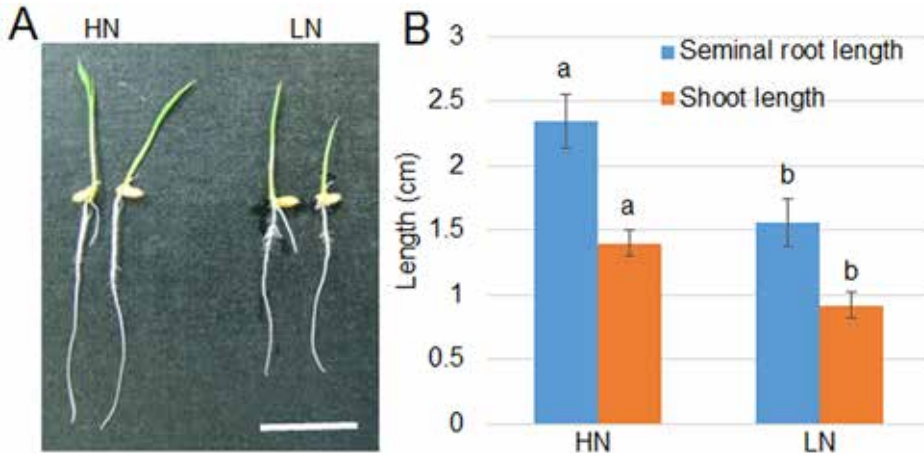

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ate susceptibility (Fig. 1B, C). These results indicate that different N sources have different effects on rice defense against sheath blight disease.

As plants cultured in N-free medium significantly promoted rice resistance to sheath blight disease, plant growth was monitored in LN and HN culture media. Rice seedlings were grown in HN or LN for 5 days, and the root and shoot lengths were measured. The data showed that seedling size was obviously larger under HN culture conditions than LN culture conditions (Fig. 2B). These results showed that lower N in the growth medium might had enhanced rice resistance to sheath blight, yet plant growth was affected.

Overexpression of AMT1;2 increased N use efficiency and resistance
to sheath blight disease

To test whether modification of NH_4^+ uptake ability was also a plant defense response, *AMT1;2* overexpression lines were generated. Over 10 individual lines were generated, and 4 lines (*AMT1;2 OX1–4*) had their expression levels further analyzed using Northern blot analysis. *AMT1;2* was weakly expressed in wild-type plants, yet it was highly ex-
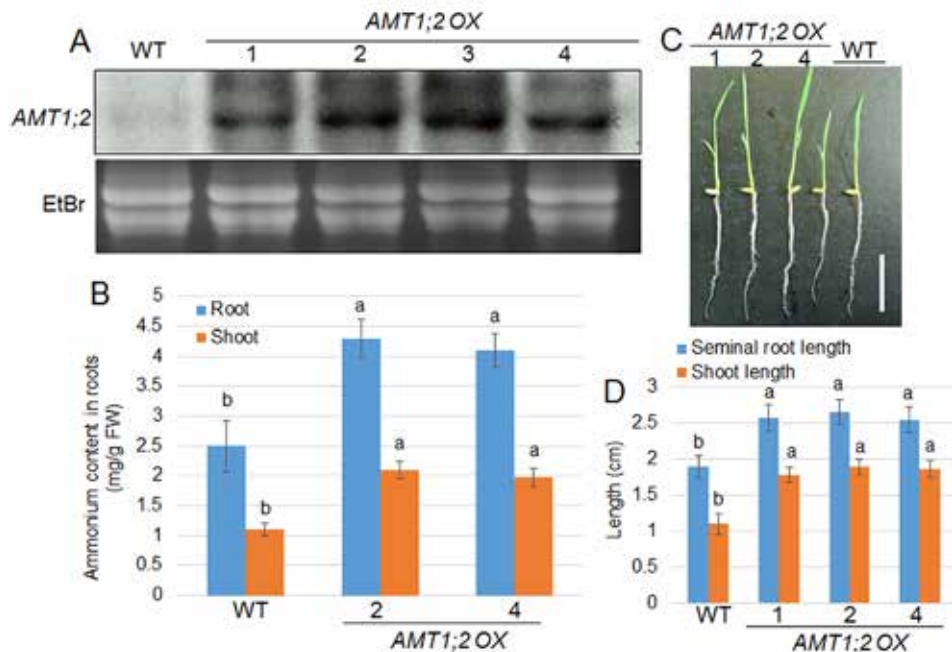

Figure 3. Cellular NH_4^+ contents and N-dependent growth of *AMT1;2* overexpressors. (A) The expression levels of *AMT1;2* was detected by Northern blot analysis using RNAs extracted from 1-week-old wild-type and *AMT1;2* overexpressors (*AMT1;2 OX1, 2, 3, 4*) seedlings. (B) Cellular NH_4^+ contents in shoots and roots of wild-type and two independent *AMT1;2 OX* lines (2 and 4) were measured. (C) Wild-type and three independent *AMT1;2 OX* lines (1, 2, and 4) grown in the modified MS solution containing 0.1 mM NH_4NO_3 for 5 days. Scale bar=1 cm. (D) The shoot and root lengths of plants shown in (C) were calculated. Different letters indicate significant differences at $P<0.05$

pressed in *AMT1;2 OX* lines. The expression levels were higher in *OX2* and *OX3* than in *OX1* and *OX4* (Fig. 3A). Next, cellular NH_4^+ contents were measured in the shoots and roots of wild-type and two *AMT1;2 OX* lines (*OX2* and *OX4*). Roots contained higher NH_4^+ than the shoots, and cellular NH_4^+ contents were much higher in *AMT1;2 OXs* than in wild-type plants (Fig. 3B). Additionally, the growth of *AMT1;2 OXs* under the LN condition was analyzed. The data showed that *AMT1;2 OXs* developed much longer shoots and roots than wild-type plants (Fig. 3C, D).
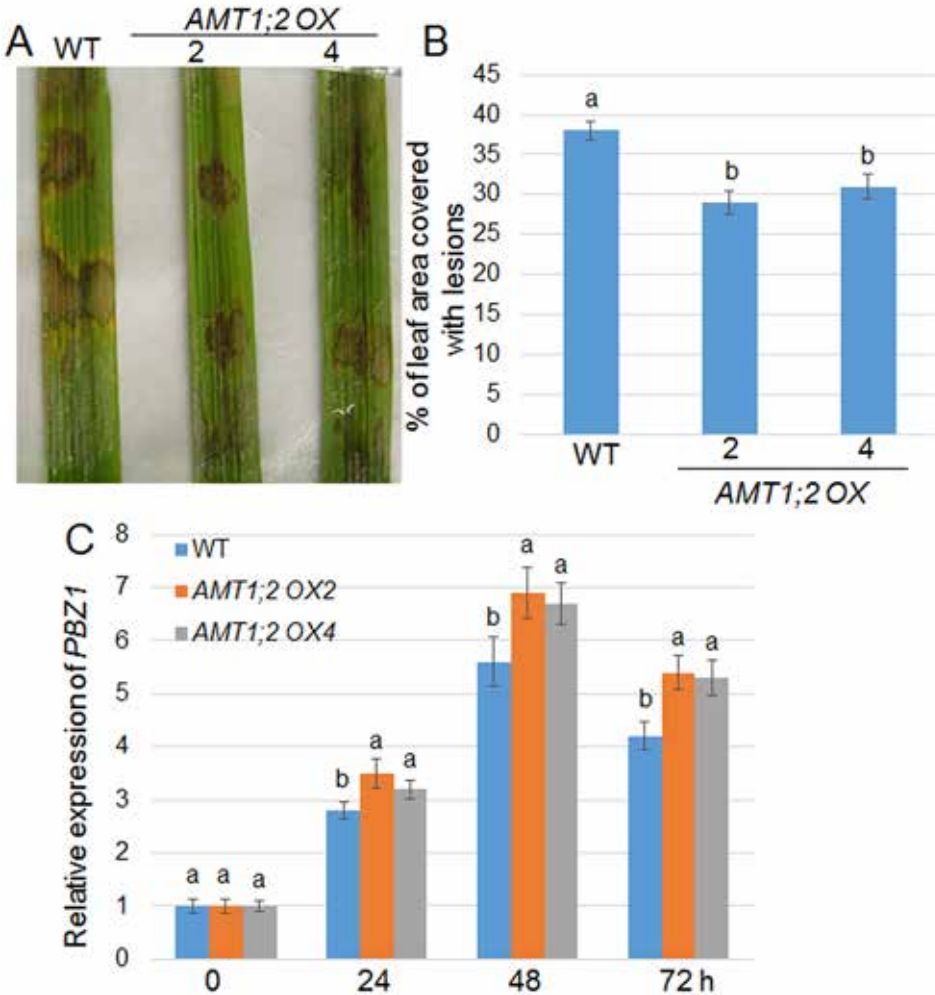

Figure 4. Response of *AMT1;2 OX* plants to *R. solani*. (A) Lesions of 3-week-old wild-type and *AMT1;2 OX* plants were photographed after 4 days of *R. solani* AG1-1A inoculation. (B) Percentage of leaf area covered with lesions shown in (A) were calculated (n > 15). (C) The 3-week-old plants were inoculated with *R. solani* AG1-1A. *PBZ1* expressions after 0, 24, 48, and 72 h of *R. solani* inoculation were examined in wild-type and two *AMT1;2 OX* lines. Different letters indicate significant differences at P < 0.05

Further, the response of *AMT1;2 OX* to *R. solani* AG1-1A was examined. The 1-month-old plant leaves were inoculated with *R. solani* and the lesion area on the leaves were calculated. The results indicated that wild-type and *AMT1;2 OXs* were susceptible to sheath blight disease, with the lesion area in the leaves of *AMT1;2 OXs* much lower than in the wild-type plants (Fig. 4A, B). The marker gene expression test showed that *PBZ1* expression was induced upon *R. solani* inoculation, and the induction folds were significantly higher in *AMT1;2 OXs* than in wild-type plants.

GS1;1 mutant is more susceptible to R. solani

As *AMT1;2 OXs* were less susceptible to *R. solani* than wild-type plants, the role of the NH_4^+ assimilation process in sheath blight disease defense was further examined. GS was the first step enzyme to catalyze the NH_4^+ assimilate into the amide group of glutamine. The *gs1;1* mutant with T-DNA inserted in the second exon was shown in Figure 5A. To
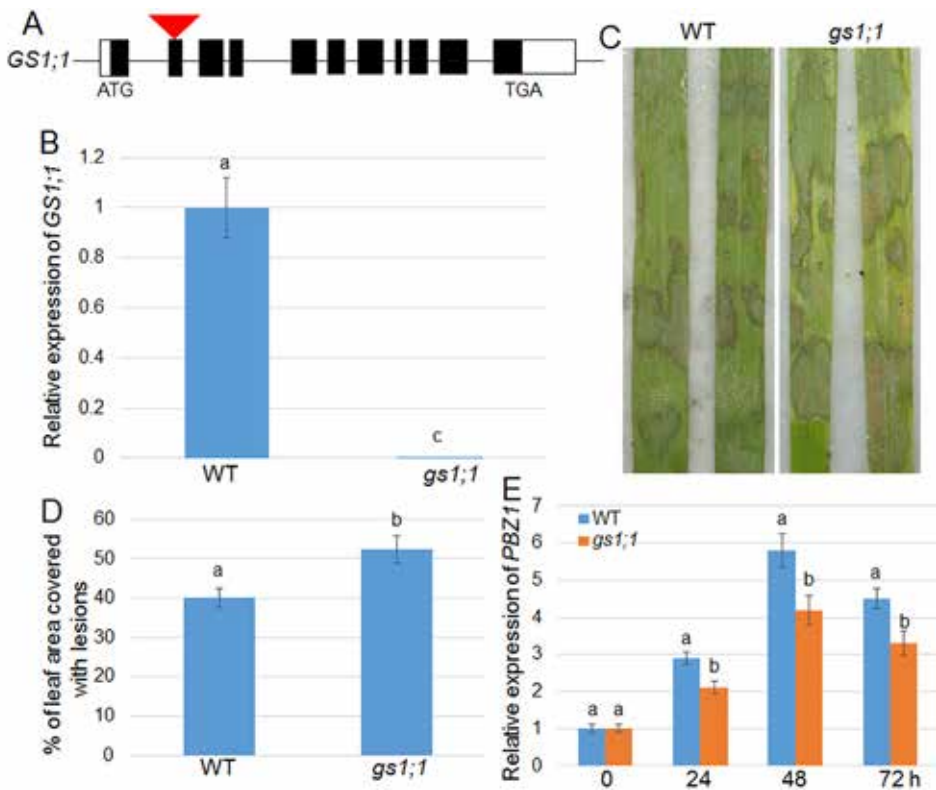

Figure 5. Response of *GS1;1* mutant to *R. solani*. (A) The diagram showed the genomic structure of the *GS1;1* T-DNA insertional mutant *(gs1;1)*. Black and white boxes indicate the exons and UTR regions, respectively. The triangle in the second exon indicates T-DNA insertion sites. (B) *GS1;1* expression levels in wild-type and *gs1;1* mutant were analyzed using qRT-PCR. (C) Lesions of 3-week-old wild-type and *gs1;1* mutant were photographed after 4 days of *R. solani* AG1-1A inoculation. (D) Percentage of leaf area covered with lesions shown in (C) were calculated (n>15). (E) *R. solani* infection-mediated *PBZ1* expression patterns in wild-type and *gs1;1* mutant were analyzed after 0, 24, 48, and 72 h of inoculation. Different letters indicate significant differences at P<0.05
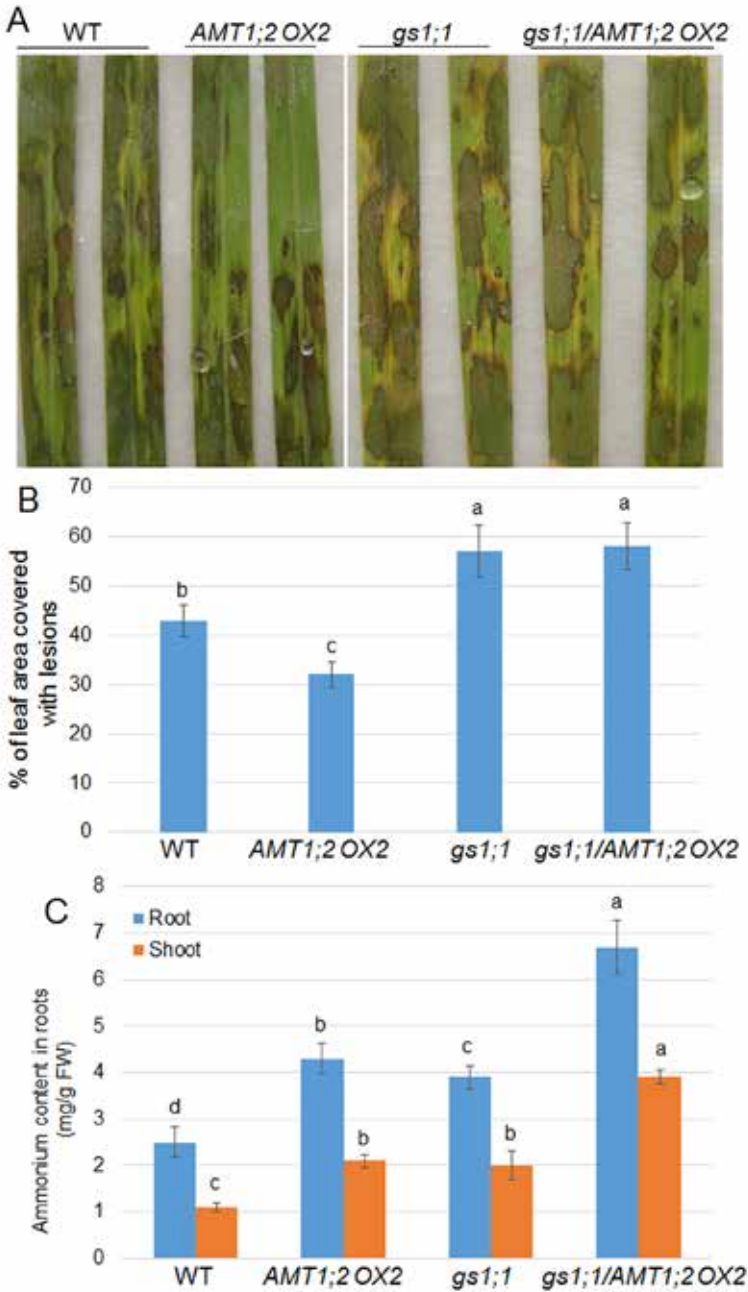
Figure 6. Effect of *GS1;1* mutation on *AMT1;2 OX* plant defense against *R. solani*. (A) Lesions of 3-week-old wild-type, *AMT1;2 OX2*, *gs1;1*, and *gs1;1/AMT1;2 OX2* mutants were photographed after 4 days of *R. solani* AG1-1A inoculation. (B) Percentage of leaf area covered with lesions shown in (A) were calculated (n>15). (C) Cellular NH_4^+ contents in shoots and roots of wild-type, *AMT1;2 OX2*, *gs1;1*, and g*s1;1/AMT1;2 OX2* were measured. Different letters indicate significant differences at P<0.05

verify whether it was a knock-out mutant, qRT-PCR analysis was performed, and the results showed that no visible transcript was detected in the *gs1;1* mutant (Fig. 5B). The response of *gs1;1* to *R. solani* AG1-1A was evaluated. The 1-month-old wild-type and *gs1;1* mutant leaves were inoculated with *R. solani*, and the lesion area on the leaves calculated. The results showed that *gs1;1* was more susceptible to *R. solani* than the wild-type control (Fig. 5C, D). The *PBZ1* expression patterns were further examined after *R. solani* inoculation. The qRT-PCR data showed that *PBZ1* levels were similar in the wild-type and *gs1;1* before *R. solani* inoculation, and *PBZ1* was less induced in *gs1;1* than in the wild-type after inoculation (Fig. 5E).

AMT1;2 OX plants are relatively resistant to R. solani *and require gs1;1 activity*

AMT1;2 OX plants exhibited less susceptible symptoms to *R. solani* infection than *gs1;1* plants that were more susceptible than the wild-type controls. Therefore, we examined whether the *AMT1;2 OX* influence on *R. solani* defense depends on the NH_4^+ assimilation process or not. To investigate this hypothesis, the genetic combination between *AMT1;2 OX2* and *gs1;1* was generated and *R. solani* response was evaluated. The results indicated that segregated *AMT1;2 OX2* was less susceptible to sheath blight disease, whereas *gs1;1* and *gs1;1/AMT1;2 OX2* plants were more susceptible than wild-type plants to sheath blight disease (Fig. 6A, B). To specify whether NH_4^+ contents controlled the defense against *R. solani*, in the present study the cellular NH_4^+ contents were measured in the shoots and roots of wild-type, *gs1;1*, *gs1;1/AMT1;2 OX2*, and *AMT1;2 OX* seedlings. The *gs1;1* and *AMT1;2 OX2* shoots and roots accumulated higher NH_4^+ than the wild-type shoots and roots, and cellular NH_4^+ contents were higher in *AMT1;2 OX2* than in *gs1;1*. In addition, *gs1;1/AMT1;2 OX2* plants accumulated much higher NH_4^+ than *AMT1;2 OX2* and *gs1;1* plants (Fig. 6C).

Discussion

N is important for plant growth and yield production as well as for plant response to pathogens (Fagard et al. 2014). In most soils, NO_3^- and NH_4^+ represent the major forms for N uptake in higher plants. The reduction of NO_3^- to NH_4^+ consumes 12–26% of photosynthetically-generated reductants. Therefore, the use of NH_4^+ as a N source conserves a large amount of energy for plants (Bloom 1997; Noctor and Foyer 1998; Patterson et al. 2010). Because of the higher abundance of NH_4^+ in paddy soils, NH_4^+ nutrition is of particular importance for the production of rice plants. In the present study, we cultured rice plants with NO_3^-, NH_4^+, and glutamine as the sole N source or under N-free conditions. Further, *R. solani* AG1-1A was inoculated to examine the role of different N nutrition on sheath blight disease. The data indicated that N starvation significantly increased resistance to sheath blight disease, whereas glutamine feeding severe the disease index. NO_3^- treatment had a weak effect whereas NH_4^+ nutrition exhibited a moderate effect on sheath blight disease (Fig. 1), suggesting that different N sources had different effects on defense responses. We speculate that different N sources might act as signal molecules to modu-

late their signals or that N assimilation is important for rice defense. Previous reports have indicated that glutamate treatment in rice plants increased resistance to blast disease (Kadotani et al. 2016); however, our results indicated that amino acids played different roles in sheath blight disease defense.

N starvation to plants obviously enhanced rice resistance to sheath blight disease; however, it significantly affected plant growth with shorter shoot and root lengths (Fig. 2). *AMT1;1* overexpression increased N use efficiency, developed bigger plants, and increased rice yield under limited NH_4^+ in growth medium (Ranathunge et al. 2014). *AMT1;2* overexpressing plants developed longer roots and shoots under LN condition (Fig. 3). In addition, *AMT1;2* overexpression promoted rice resistance to sheath blight disease, suggesting that activation of NH_4^+ uptake activated plant immunity. Marker gene *PBZ1* expression was similar in wild-type and *AMT1;2 OX* seedlings; however, the induction folds were higher in *AMT1;2 OX* than in wild-type plants, indicating that *AMT1;2* overexpression activated defense-related genes in a pathogen dependent manner. *AMT1;2* overexpression increased cellular NH_4^+ contents. The *GS1;1* mutant (*gs1;1*) has been reported previously to accumulate less amino acids and severely affect rice yield production (Tabuchi et al. 2005). To specify whether the activation of the defense system depended on cellular accumulation of NH_4^+ or assimilation products, the response of *GS1;1* mutant (*gs1;1*) to sheath blight disease was examined. The *gs1;1* mutant was more susceptible to *R. solani* infection than the wild-type control; however, the *GS1;1* mutation accumulated more cellular NH_4^+, suggesting that cellular NH_4^+ contents were not associated with sheath blight disease.

AMT1;2 OX plants exhibited less susceptible symptoms to sheath blight disease, whereas the *gs1;1* mutant was more susceptible to sheath blight disease. To determine whether *AMT1;2 OX* phenotype was associated with the NH_4^+ assimilation process, *AMT1;2 OX and gs1;1* mutants were crossed and the double mutant response to *R. solani* was examined. The results indicated that *AMT1;2 OX* phenotype against *R. solani* disappeared when *GS1;1* was mutated, and cellular NH_4^+ contents were much higher than in *AMT1;2 OX* and *gs1;1*. This result strongly suggested that *AMT1;2 OX* might be less susceptible to sheath blight disease because of the accumulation of more metabolites rather than increased cellular NH_4^+ contents. Exogenous feeding of N nutrition accelerated rice sheath blight disease. In tomato, exogenously treated with NH_4^+, activated accumulation of reactive oxygen species levels in the cells was found to protect tomatoes against *Ps. Syringae* (Fernandez-Crespo et al. 2015). However, overexpression of *AMT1;2* was less susceptible to sheath blight disease, implying that treatment of higher concentrations of N might result in an overactivation of its signal or accumulation of metabolites. *AMT1;2* overexpression increased N use efficiency with better development of plants under limited N conditions, which might more efficiently uptake and utilize N sources and produce moderate levels of metabolites. Our study identified new evidence that overexpressing *AMT1;2*, an NH_4^+ transporter gene, increased N use efficiency as well as resistance against sheath blight disease. These results might be useful to utilize in rice molecular breeding.

Acknowledgements

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Electronic Supplementary Material (ESM)

Electronic Supplementary Material (ESM) associated with this article can be found at the website of CRC at https://akademiai.com/loi/0806

Electronic Supplementary *Table S1*. Primer sequences