BZR1 Regulates Brassinosteroid-Mediated Activation of AMT1;2 in Rice

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Although it is known that brassinosteroids (BRs) play pleiotropic roles in plant growth and development, their roles in plant nutrient uptake remain unknown. Here, we hypothesized that BRs directly regulate ammonium uptake by activating the expression of rice AMT1-type genes. Exogenous BR treatment upregulated both AMT1;1 and AMT1;2 expression, while this induction was impaired in the BR-receptor gene BRI1 mutant d61-1. We then focused on brassinazole-resistant 1 (BZR1), a central hub of the BR signaling pathway, demonstrating the important role of this signaling pathway in regulating AMT1 expression and rice roots NH4+ uptake. The results showed that BR-induced expression of AMT1;2 was suppressed in BZR1 RNAi plants but was increased in bzr1-D, a gain-of-function BZR1 mutant. Further EMSA and ChIP analyses showed that BZR1 bound directly to the BRRE motif located in the promoter region of AMT1;2. Moreover, cellular ammonium contents, 15NH4+ uptake, and the regulatory effect of methyl-ammonium on root growth are strongly dependent on the levels of BZR1. Overexpression lines of BRI1 and BZR1 and Genetic combination of them mutants showed that BZR1 activates AMT1;2 expression downstream of BRI1. In conclusion, the findings suggest that BRs regulation of NH4+ uptake in rice involves transcription regulation of ammonium transporters.

Keywords: brassinosteroids, ammonium uptake, BZR1, AMT1;2, rice

INTRODUCTION

Inorganic nitrogen (N) is an important plant nutrient and is absorbed from the rhizosphere in two forms, nitrate and ammonium. In the paddy field, high levels of NH4+ are of particular importance to the rice yield. Further understanding of the molecular basis and regulation of ammonium transport and its translocation to buds is needed to promote efficient nitrogen absorption and to improve crop yields. Ammonium transporter (AMT) proteins can induce high-affinity NH4+ uptake from the rhizosphere to root cells, and the transporter AMT2;1 has also been shown to play a crucial role in ammonium root-to-stem translocation (Giehl et al., 2017). Sequence analysis has identified 10 AMT members in the rice genome (Suenaga et al., 2003; Loque and von Wirén, 2004). Of these, AMT1;1, AMT1;2, and AMT1;3 are the main three AMTs. The expression patterns of these three proteins differ between different tissues with AMT1;2 and AMT1;3 mainly expressed in plant roots, while AMT1;1 is constitutively expressed in different tissues (Sonoda et al., 2003a). In rice, overexpression of AMT1;1 increases NH4+ uptake, improves plant growth and promotes yield production in limited N-fertilization conditions (Ranathunge et al., 2014).
However, the overexpression of AMT1;3 has an opposite effect in regulating rice growth and NH$_4^+$ uptake to AMT1;1 (Bao et al., 2015). Studies have shown that transcriptional regulation of AMT genes strongly influences the plant's N content and uptake of different forms of externally applied N. NH$_4^+$ can upregulate the expression of both AMT1;1 and AMT1;2, and inhibit the expression of AMT1;3. Under N starvation conditions, AMT1;3 is upregulated in response to NH$_4^+$ (Kumar et al., 2003; Sonoda et al., 2003a). Similar regulation at the posttranscriptional and posttranslational levels have also been observed in Arabidopsis. For example, AMT1;1 has been shown to be phosphorylated in C-terminal threonine residue to inhibit transporter activity in an NH$_4^+$-dependent manner (Yuan et al., 2007; Lanquar et al., 2009), and further, CBL-interacting serine/threonine protein kinase 23 (CIPK23) was reported to phosphorylate AMT1 to inhibit ammonium uptake (Straub et al., 2017). Indeterminate domain 10 (IDD10), a transcription factor, has recently been shown to directly activate AMT1;2 in rice (Xuan et al., 2013), furthermore, the ABI3/VP1 transcription factor RAV1 activates AMT1;2 to directly modulate NH$_4^+$ uptake in rice (Xuan et al., 2016).

Transcriptome studies using gain-of-function mutants of the BES1 transcription factor and wild-type plants have identified the Arabidopsis genes regulated by brassinosteroids (BRs). These genes included AtAMT1;1 which was found to be upregulated by BR signaling activation (Godha et al., 2004; Yu et al., 2011). BRs are important phytohormones that bind to the cell surface receptor Brassinosteroid Insensitive 1 (BRI1), initiating a signaling cascade in which BRI1 binds to BRII-Associated Receptor Kinase 1 (BAK1) leading to the downstream inactivation of the kinase Brassinosteroid Insensitive 2 (BIN2). The protein phosphatase PP2A dephosphorylates two master transcription factors Brassinazole-Resistant 1 (BZR1) and BR1-EMS-Suppressors 1 (BES1), and the non-phosphorylated BZR1 and BES1 translocate to the nucleus to regulate the expression of BR responsive genes (Li and Chory, 1997; Li et al., 2002; Nam and Li, 2002; Kim and Wang, 2010; Yang et al., 2011; Guo et al., 2013; Tong and Chu, 2018). In addition, the stunning and BR-insensitive phenotype of bRI1 BR receptor mutants can be rescued by the enhanced stability of BES1 and BZR1 in bRI1-D and bbr1-D gain-of-function mutants (Wang et al., 2002; Yin et al., 2002). In rice, RAV1, an upstream component of BR signaling, regulates BR homeostasis through binding to an E-box motif in the promoter regions of the BR receptor and biosynthesis genes (Je et al., 2010). BRs treatment enhances the expression of AMT1;1 and AMT1;2 in rice (Xuan et al., 2016); however, the detailed mechanism remains obscure.

These findings raise the questions of whether BRs play a role in nutrient uptake, and whether there is a direct regulatory link between BZR1 and AMT1's in rice. In this study, we first analyzed the expression of AMT1 genes affected by BRs in the key BR signal transcription factor BZR1 and the BR receptor gene BRII1 mutants. We then investigated the expression patterns of AMT1 family members in the roots of the BRI1 mutant d61-1, bbr1-D siblings, as well as the BZR1 knockdown BZR1 RNAi. Genetic combinations between BRI1 and BZR1 were generated to examine activation of BRI1 and BZR1 in BR-mediated induction of AMT1;2. In addition, the cellular ammonium contents and 15N abundance were tested to investigate BZR1 function in ammonium uptake. Taken together, our results showed that BR-dependent ammonium uptake is partially controlled by BZR1.

**MATERIALS AND METHODS**

**Plant Materials and Growth Conditions**

The coding sequence of BZR1 was cloned into the pCambia1302 vector to construct the BZR1-GFP-expressing plasmid. The pCambia1302-BZR1 vector was subsequently transformed into Nipponbare rice calli to generate BZR1-GFP transgenic lines. The bRI1-D in the Dongjin background as well as d61-1, bbr1-D, and BZR1 RNAi in the Nipponbare background were described previously (Yamamuro et al., 2000; Jeong et al., 2002; Bai et al., 2007; Qiao et al., 2017). BZR1 RNAi, d61-1, bbr1-D, bbr1-D/bZRI RNAi, BZR1-GFP, and d61-1/bbr1-D plants were grown in the greenhouse. Plants were first grown in distilled water (dH$_2$O) for 1 week and subsequently transferred to brassinolide (BL) solution for analyzing BR effects on AMT1 expressions. Whole roots were harvested after 3 h of BL treatment. To examine the effects of NH$_4^+$ on the expression of BZR1, plants were grown in dH$_2$O for 2 weeks before transfer to N-free nutrient solution for a further 3 days of growth (Abiko et al., 2005). The plants were then grown in the nutrient solution containing 0.5 mM (NH$_4$)$_2$SO$_4$ at pH 5.5. The roots were sampled at 0 and 3 h after the transfer. To test the effects of methyl-ammonium (MeA) on root growth, we added 1 mM KNO$_3$ as the only source of N and different concentrations of MeA to 0.5 × MS medium, and cultivated wild-type, BZR1 RNAi, and bbr1-D plants in the modified medium. The primary root length was measured and recorded on the sixth day.

**RNA Extraction and Quantitative RT-PCR Analysis**

Cellular total RNA was isolated by using the TRIZol reagent (Takara, Dalian, LN, China), and the RNA was treated with RNase-free DNase (Promega, Madison, WI, United States) to eliminate genomic DNA contamination. The GoScript Reverse Transcription kit (Promega, Madison, WI, United States) was used to synthesize cDNA. Quantitative RT-PCR was performed using the Illumina Research Quantity software, Illumina Eco 3.0 (Illumina, San Diego, CA, United States), and each gene expression was normalized against that of the Ubiquitin level. The primers used for qRT-PCR are listed in Table 1.

**ChIP Assay**

Rice calli (8 g) expressing 35S: BZR1:GFP, and 35S:GFP were used for the ChIP assay. A pre-immune serum was used for pre-absorption before immunoprecipitation, and an anti-GFP monoclonal antibody (Clontech, Takara Bio, Japan) was used for immunoprecipitation. The immunoprecipitated DNAs were analyzed by ChIP-PCR for identification of the BZR1 binding region. The immunoprecipitated DNA was normalized by each input DNA in ChIP-PCR (Je et al., 2010). The primers used for the ChIP-PCR are shown in Table 1.
TABLE 1 | Primer sequences used in this study.

| Primer | Sequence |
|--------|----------|
| Ubiquitin F | CACGGTCTCAACAACTCAG |
| Ubiquitin R | TGAAGACCCCTGACGAGGAAAG |
| AMT1;1 F | AGTACGTCGAGGAGATCTAC |
| AMT1;1 R | ACCTGCTTGCTCGATTGG |
| AMT1;2 F | TAGACATGCGCTCCATCTC |
| AMT1;2 R | TAACGATGCTTTCATGTTG |
| AMT1;3 F | AGAGGATGCGAGCTGATC |
| AMT1;3 R | CCTGCTGCTCCGAGCTTCTTGG |
| AMT1;2 P-F | GGTGCGCATCGTCGTGAGCTGCTATTAG |
| AMT1;2 P-R | GAAGGCGCGTCAACAGAGACTGTGA |
| BZR1 RT-F | GGATTCCGCTTCGCGCCGACGCCGAGCGTGAG |
| BZR1 RT-R | CTCGGCGTCGGCGCGAATGA |
| AMT1;2 P-F | GCTCGCGGGATGGCGATGCGCGCTC |
| AMT1;2 P-R | TAAGCATGATGTTCATGGTG |
| AMT1;2 F | TAGACATGGCCTCCCATCTC |
| AMT1;1 P-F | GATCGTTCGTTCTGGATTG |
| AMT1;1 P-R | AGTACGTCGAGGAGATCTAC |
| Ubiquitin R | TGAAGACCCTGACTGGGAAG |
| Ubiquitin F | CACGGTTCAACAACATCCAG |

Electrophoretic Mobility Shift Assay (EMSA)

BZR1 ORF sequences were sub-cloned into the pET28a (+) expression vector to produce His:BZR1 recombinant protein in Escherichia coli strain BL21 DE3 after 4 h of 0.5 mM IPTG treatment at 28°C. To perform EMSA, 1 μg of His:BZR1 protein and 40k cpm of the 32P-labeled DNA probes were used. The protocol was followed as previously described (Je et al., 2010). Primers used in the EMSA are listed in Table 1.

Transcriptional Activity Analysis

The effector (35S:BZR1), reporters [pAMT1;2 and BRRE (BR Responsive Element)-mutated promoter mpAMT1;1-GUS fusions] and an internal control (35S:LUC) were co-transformed into protoplasts from Arabidopsis for testing transcriptional activation (Yamaguchi et al., 2010). 35S:BZR1 was cloned into the GALABD region of the p35S:GALABD vector and 2.5 kb of normal and BRRE-mutated (in which CTGTG^TC was replaced by TTTTTTTT) AMT1;2 promoters were cloned into the TATA region of the p35S:TATA:GUS vector (Xuan et al., 2013). PEG-mediated transformation and subsequent activity measurement were performed as previously described (Yoo et al., 2007).

Determination of Intracellular Ammonium Contents

Cellular ammonium contents in rice roots were calculated by using an F-kit (Roche, Basel, Switzerland) following the manufacturer’s instructions (Oliveira et al., 2002).

15N Uptake Analysis

Wild-type, BZR1 RNAi, d61-1, bri1-D, bri1-D, bri1-D/BZR1 RNAi, and d61-1/bri1-D plants were cultivated for a 2-week nursery period in deionized water, following which the seedlings were transferred to N-free nutrient solution to continue culturing (Sonoda et al., 2003b). After culturing for 3 days, the protoplasmic absorption of 15NH4+ was analyzed. The detailed method for the calculation of 15NH4+ influx and the ratio of 15N to 14N in the total N pool was as previously described (Xuan et al., 2016).

Statistical Analysis

Statistical analysis was performed with Prism 5 software (GraphPad, San Diego, CA, United States). All data were expressed as mean ± SE. Comparison between multiple groups was performed by using one-way ANOVA with values of P < 0.05 considered as significant, followed by Bonferroni’s multiple comparison tests.

RESULTS

BR Treatment Induces BRI1-Dependent AMT1 Transcription

Brassinolide is the most active form of BR. To investigate whether BR affects the expression of rice AMT1, we used a series of BL concentration gradients of 0, 10, 100, and 200 nm to treat the wild-type, a weak allele of rice BRII1 mutant, d61-1, and the BRII1 overexpression line bri1-D (Jeong et al., 2002), and compared the changes in the AMT1 expression level under the different treatment conditions. Quantitative RT-PCR analysis showed different patterns in the response of the AMT gene to BR at the transcriptional level: AMT1;1 and AMT1;2 showed dose-dependent upregulation in response to BR treatment, while no obvious change was observed in AMT1;3. Without BR treatment, the transcription levels of AMT1;2 and AMT1;3 in the d61-1 mutant were slightly lower than those of the control group, while in bri1-D, the transcription levels of these genes were higher than those of wild-type plants. Among the various genotypes treated with BR, the expression levels of AMT1;1 and AMT1;2 in wild-type plants were higher than the level of d61-1. The expression level of AMT1;1 and AMT1;2 in bri1-D was significantly higher than that of the wild-type plants. Also, expression of a BR biosynthetic gene D2 was analyzed. The result indicated that...
D2 expression was suppressed by BR treatment in a dose-dependent manner, and D2 expression level was higher in d61-1 while lower in bri1-D compared to wild-type plants (Figure 1). These results suggest that BR mediates the regulation of AMT1;1 and AMT1;2 transcription levels via the cell surface receptor BRI1.

**BR-Mediated Induction of AMT1;2 Depends on BZR1**

As in Arabidopsis, rice BZR1 has been reported to be a key BR signaling transcription factor controlling the expression of downstream genes (Bai et al., 2007). To investigate the role of BZR1 in BR-mediated AMT1 induction, BZR1 RNAi (#1), the knockdown transgenic line, and bzr1-D dominant mutant line were constructed (Bai et al., 2007; Ren et al., 2020). The changes in AMT1;1 and AMT1;2 transcription levels in these lines after BL treatment were monitored. The transcriptional abundance of AMT1;1 was not affected by changes in BZR1 levels but could be increased in response to BL treatment in all plants (Figure 2A). Although the changes in the AMT1;2 transcription level in the BZR1 RNAi mutants and bzr1-D were not significantly associated with BL treatment, they showed a significant decrease and increase, respectively. Only the AMT1;2 expression levels correlated with the BZR1 level in a BR-dependent manner (Figure 2B). To verify BZR1 levels in the BZR1 RNAi plants, qRT-PCR was performed. The results indicated that BZR1 levels were reduced by about 60–70% in BZR1 RNAi lines (#1–#4). Also, AMT1;2 expression levels were significantly lower in the BZR1 RNAi lines (#1–#4) than in the wild-type plants (Figure 2C).

**BZR1 Directly Binds to the Promoter to Activate AMT1;2 Expression**

Since the changes in the transcription level of AMT1;2 are highly consistent with the changes in the expression level of BZR1, which indicated that BZR1 acts as a transcriptional activator upstream of AMT1;2. To verify whether the binding site of BZR1 includes the promoter of AMT1;2, we constructed transgenic plants of 35S:GFP and 35S:BZR1:GFP and performed ChIP assays. Promoter-sequence analysis showed that two BRRE (BR Responsive Element) motifs were located within a 2.5 kb stretch in front of the AMT1;2 start codon (Figure 3A). We designed four primer pairs to amplify the four fragments (P1−P4) of the AMT1;2 promoter and performed qPCR experiments to check the GFP-immunoprecipitates in 35S:GFP and 35S:BZR1:GFP transgenic siblings (Figure 3B). The ChIP results showed that BZR1 could bind directly to the AMT1;2 promoter, especially in the P4 region. Since the P4 fragment harbors two putative BRRE motifs (Figure 3A), we further performed EMSA experiments to determine which of the BRRE motifs was responsible for the BZR1 binding. We designed two specific probes each containing a BRRE motif. The B probe showed stronger binding to BZR1 (Figure 3A) while the binding of the A probe was slightly weaker. When the probes were mutated, their binding to BZR1 was lost. These results suggest that BZR1 can bind both BRRE motifs in the P4 region but the binding strength is different (Figure 3C).

In the current study, we identified two cis-elements targeted by the promoter of BZR1 which are closely related to transcriptional activation of AMT1;2. To further verify whether these cis-elements have similar functions in vivo, we used the Arabidopsis protoplast system to perform transient expression assays (Figure 3D). We also used the 35S:BZR1 plasmid and a vector expressing GUS, which is strictly controlled by one
of four different types of 2.5 kb AMT1;2 promoters namely, the native (pWT) and three mutated (pmP4) promoters. In Arabidopsis protoplasts, the 35S:BZR1 plasmid and the GUS vector are co-transformed into a vector expressing GUS. In these mutated promoters, BRRE motif sequences (CGTG\^7/T\^C) were observed to be replaced by the sequence TTTTTT. To eliminate the error caused by conversion efficiency, 35S:LUC was used as an internal reference in each assay. By comparing the activity of the GUS genes under different promoter-driven conditions, we found that the GUS activity in the wild-type plants was approximately twice that driven by the reporter gene 35S:LUC. Thus, the error caused by conversion efficiency, 35S:LUC was used as an internal reference in each assay. By comparing the activity of the GUS genes under different promoter-driven conditions, we found that the GUS activity in Arabidopsis protoplasts was approximately twice that driven by the reporter gene alone under the promoter pWT-driven conditions. Conversely, no detectable GUS activity was observed in the protoplasts which were controlled by the mutant promoter (pmP4) (Figure 3D). These results indicated that direct combination with BZR1 is necessary for AMT1;2 promoter activation.

**BZR1 Affects the Absorption of NH\textsubscript{4}\textsuperscript{+} by Plant Roots**

Root absorption of NH\textsubscript{4}\textsuperscript{+} is one of the main ways for plants to obtain N nutrients. To demonstrate whether BZR1 plays a role in this process, \textsuperscript{15}N-labeled ammonium was used to determine the efficiency of N absorption. The NH\textsubscript{4}\textsuperscript{+} concentrations in the root tissues of the wild-type, BZR1 RNAi, and bzr1-D lines were also determined. Seventeen day-old hydroponic seedlings were soaked in 200 \textmu M of \textsuperscript{15}NH\textsubscript{4}\textsuperscript{+} solution for 6 min, after which the short-term import rate of the \textsuperscript{15}N-labeled ammonium in the plant roots was determined. Expressing the \textsuperscript{15}N influx in \textmu moles g\textsuperscript{-1} root dry weight h\textsuperscript{-1} (Yuan et al., 2007, 2013), the \textsuperscript{15}NH\textsubscript{4}\textsuperscript{+} influx in the BZR1 RNAi plants was only 68% of that of the wild-type, while the influx of \textsuperscript{15}NH\textsubscript{4}\textsuperscript{+} in the bzr1-D plants was greater than that of the wild-type plants (Figure 4A). We introduced the concept of “\textsuperscript{15}N abundance,” representing the proportion of \textsuperscript{15}N to \textsuperscript{14}N in the total N pool, to further explore the role of BZR1 in the short-range transport of NH\textsubscript{4}\textsuperscript{+}. Compared with the \textsuperscript{15}N internal flow results, the \textsuperscript{15}N abundance in the total \textsuperscript{15}N was lower in the BZR1 RNAi and higher in the bzr1-D plants compared to the wild-type (Figure 4B). The results of these short-term \textsuperscript{15}N absorption experiments indicate that BZR1 plays an important role in mediating NH\textsubscript{4}\textsuperscript{+} influx.

BZR1 can regulate the expression of AMT1;2 genes in plant roots. We suspect that this regulation may be related to the long-term transport process of NH\textsubscript{4}\textsuperscript{+} in the roots. Therefore, the NH\textsubscript{4}\textsuperscript{+} content in the rice roots of 3-day-old wild-type, BZR1 RNAi, and bzr1-D seedlings grown on 0.5 × MS medium was determined. As expected, the content of NH\textsubscript{4}\textsuperscript{+} in the roots of BZR1 RNAi was lower than in the wild-type seedlings, while the roots of bzr1-D plants contained more NH\textsubscript{4}\textsuperscript{+} than the wild-type plants (Figure 4C). Furthermore, a toxic ammonium analog, MeA, was used as a replacement addition to the NH\textsubscript{4}\textsuperscript{+}-free medium using a concentration gradient. Wild-type, BZR1 RNAi, and bzr1-D plants were grown in this medium and the length of the plant's initial rooting was measured after 6 days of growth. In the absence of MeA, the primary roots of BZR1 RNAi were shorter than those of the wild-type and bzr1-D. With MeA treatment, the BZR1 RNAi root length was similar to that of the wild-type, while the bzr1-D roots were significantly shorter, the shortest in length among the three genetic lines (Figures 4D,E). These observations illustrated that the BZR1 RNAi response to MeA treatment is weaker than that of wild-type, while bzr1-D sensitivity to MeA treatment is relatively higher. Therefore, it can be inferred that BZR1 can participate in the process of rice root absorption of NH\textsubscript{4}\textsuperscript{+} in a long-term manner by regulating the expression of AMT1;2 genes.

**BZR1 Regulates AMT1;2 at the Downstream of BRI1**

As the expression of AMT1;2 genes mediated by BR is inhibited in both the d61-1 and BZR1 RNAi mutants, it is reasonable to speculate that BZR1 and BRI1 may affect the expression of AMT1;2 genes. To investigate this, a series
of genetic analyses were employed: d61-1 and bzr1-D, BZR1 RNAi, and bri1-D lines were individually hybridized and two genetic combinations were constructed. We then measured AMT1;2 levels of than the wild-type in both lines (Figure 5A). In d61-1/bzr1-D were observed in both the or without BL treatment. High levels of AMT1;2 were determined. We observed a similar low-level expression of BZR1 RNAi in bri1-D, while those in d61-1 were lower (Figure 6A). Furthermore, the bri1-D lines showed high levels of AMT1;2 mRNA, with reduced AMT1;2 mRNA expression in BZR1 RNAi, regardless of NH$_4^+$ treatment. Nevertheless, similar patterns of mRNA expression of AMT1;2 were observed in BZR1 RNAi and bri1-D/BZR1 RNAi (Figure 6B). Taken together, these results indicated that AMT1;2 expression was sensitive to the BZR1 level and that BZR1 acts downstream of BRI1 in this signaling pathway.

To determine the NH$_4^+$-dependent AMT1;2 gene expression, we treated 17-day-old seedlings originally grown in dH$_2$O and N-free nutrient medium with a 0.5 mM (NH$_4$)$_2$SO$_4$ solution. The whole roots of the plant were then collected at 0 and 3 h after treatment. To examine whether BZR1 and BRI1 play the same role in NH$_4^+$-dependent AMT1;2 as in BR-dependent induction, BZR1 and BRI1 mutants were used to examine the AMT1;2 expression levels. When compared with wild type, both bzr1-D and d61-1/bzr1-D contained higher mRNA levels of AMT1;2, while those in d61-1 were lower (Figure 6A). Furthermore, the bri1-D lines showed high levels of AMT1;2 mRNA, with reduced AMT1;2 mRNA expression in BZR1 RNAi, regardless of NH$_4^+$ treatment. Nevertheless, similar patterns of mRNA expression of AMT1;2 were observed in BZR1 RNAi and bri1-D/BZR1 RNAi (Figure 6B). Taken together, these results indicated that AMT1;2 expression was sensitive to the BZR1 level and that BZR1 acts downstream of BRI1 in this signaling pathway.
lines of evidence that support this point. First, determination of ammonium uptake and BR signaling pathways. There are three response mediated by BR. AMT1;2 promoters. This regulation occurs after sensing BR signals. Thus, AMT1;2 binding between the transcription factor BZR1 and 4 with the phenotype of higher NH4+ levels of BR susceptibility in all these genes which showed regulation of BR homeostasis were modulated. We found high uptake by the roots of rice lines, which genes involved in the process of root ammonium absorption may be coordinated with a link between BRs and AMT1;2 expression suggests that the process of root ammonium absorption may be coordinated with the physiological function of BRs in growth stimulation. In this study, we verified this issue by assessing the transcriptional levels of the AMT genes and the role they playing in ammonium uptake by the roots of rice lines, which genes involved in the regulation of BR homeostasis were modulated. We found high levels of BR susceptibility in all these genes which showed upregulated expression in response to BR treatment, especially the NH4+ transporter gene AMT1;2. Combining this result with the phenotype of higher NH4+ uptake, it can be inferred that this BR-dependent regulation is mediated by the direct binding between the transcription factor BZR1 and AMT1;2 promoters. This regulation occurs after sensing BR signals. Thus, AMT1;2-mediated uptake of NH4+ can be seen as a physiological response mediated by BR.

BZR1 plays an important role in the coordination of ammonium uptake and BR signaling pathways. There are three lines of evidence that support this point. First, determination of the transcription level of AMT1 genes in plants treated with exogenous BL showed that the AMT1;2 in roots can respond to BL processing, increasing the level of transcription. BZR1 regulation plays a vital role in this process. Since the AMT1;2 transcription level in BZR1 RNAi seedlings was also lower than that of the wild-type while the transcription level in the bzr1-D plants was higher; this expression pattern was also observed without BL treatment. Second, there was also a correlation between higher AMT1;2 transcription levels and higher NH4+ uptake. We measured the rate of uptake of 15N-labeled NH4+ by rice roots expressing different BZR1 levels. NH4+ uptake rates were compared at 15N abundance and 15N influx levels, and it was found that the NH4+ uptake of BZR1 RNAi was significantly lower than that of the wild-type, which may be explained by down-regulation of AMT1;2. This concept is further supported by the findings in bzr1-D roots, where the enrichment of 15NH4+ was higher than that in the wild-type. Therefore, in the presence of ammonium, a non-significant increase in NH4+ influx may be related to the down regulation of AMT mRNA levels (Yuan et al., 2007) or AMT protein activity (Lanquar et al., 2009; Yuan et al., 2013) at posttranscriptional or posttranslational levels. Furthermore, increased BZR1 expression levels were also associated with increased ammonium abundance in roots. We observed that all these NH4+ uptake-related traits were affected in these lines with or without additional BR treatment. This observation suggests that BZR1 is a positive regulator of NH4+ uptake under any condition conducive to plant growth. To sum up, BZR1 is the coordination center between NH4+ absorption and general

**FIGURE 4** BZR1 effects on NH4+ uptake in plants. (A) The wild-type, BZR1 RNAi and BZR1 constitutively active bzr1-D plants were hydro-cultured in deionized water for 2 weeks, and then grown in a nitrogen-free nutrient solution for 3 days. After exposure to 200 μM 15N-labeled NH4+, the absorption of ammonium by rice roots was measured. Bars represent means ± SD (n = 6). (B) The wild-type, BZR1 RNAi, and bzr1-D plants were hydro-cultured in deionized water for 2 weeks, and then grown in a nitrogen-free nutrient solution for 3 days. After exposure to nutrient solution containing 200 μM 15N-labeled NH4+ for 6 min, the absorption of ammonium by rice roots and the 15NH4+ abundance in relation to the total 15N in roots was measured. Bars represent means ± SD (n = 6). (C) Intracellular NH4+ levels of wild-type, BZR1, and bzr1-D grown for 3 days in 0.5 × MS were detected in plant roots. Data represent means ± SE (n = 3). (D) Under different conditions with 0 or 2 mM MeA, wild-type, BZR1 RNAi, and bzr1-D were grown in a modified 0.5 × MS medium containing 1 mM KNO3 for 6 days. MeA treatment significantly inhibited the growth of the primary root. In the absence of MeA treatment, the BZR1 RNAi primary root was shorter than that of the wild-type and bzr1-D. After 2 mM MeA treatment, bzr1-D showed a shorter primary root than BZR1 RNAi and wild-type. (E) The primary root growth was measured from wild-type, BZR1 RNAi, and bzr1-D with or without MeA supplementation as shown in (D). Data represent means ± SE (n > 15 plants). The experiments were repeated at least three times. Different letters represent statistically significant differences (P < 0.05).

**DISCUSSION**

Although BRs are known for their pleiotropic roles in the regulation of plant growth and development, their effect on nutrient uptake is unclear. The activation of BR signaling has been shown by transcriptome analysis of BR treated wild-type or bes1-D Arabidopsis plants to positively regulate the expression of the AMT gene AtAMT1;1 (Goda et al., 2004; Yu et al., 2011). Also, the BR signaling transcription factor RAVL1 activates AMT1;2 to enhance NH4+ uptake in rice (Xuan et al., 2016). The possibility of a link between BRs and AMT1 expression suggests that the process of root ammonium absorption may be coordinated with the physiological function of BRs in growth stimulation. In this study, we verified this issue by assessing the transcriptional levels of the AMT genes and the role they playing in ammonium uptake by the roots of rice lines, which genes involved in the regulation of BR homeostasis were modulated. We found high levels of BR susceptibility in all these genes which showed upregulated expression in response to BR treatment, especially the NH4+ transporter gene AMT1;2. Combining this result with the phenotype of higher NH4+ uptake, it can be inferred that this BR-dependent regulation is mediated by the direct binding between the transcription factor BZR1 and AMT1;2 promoters. This regulation occurs after sensing BR signals. Thus, AMT1;2-mediated uptake of NH4+ can be seen as a physiological response mediated by BR.

BZR1 plays an important role in the coordination of ammonium uptake and BR signaling pathways. There are three lines of evidence that support this point. First, determination of the transcription level of AMT1 genes in plants treated with exogenous BL showed that the AMT1;2 in roots can respond to BL processing, increasing the level of transcription. BZR1 regulation plays a vital role in this process. Since the AMT1;2 transcription level in BZR1 RNAi seedlings was also lower than that of the wild-type while the transcription level in the bzr1-D plants was higher; this expression pattern was also observed without BL treatment. Second, there was also a correlation between higher AMT1;2 transcription levels and higher NH4+ uptake. We measured the rate of uptake of 15N-labeled NH4+ by rice roots expressing different BZR1 levels. NH4+ uptake rates were compared at 15N abundance and 15N influx levels, and it was found that the NH4+ uptake of BZR1 RNAi was significantly lower than that of the wild-type, which may be explained by down-regulation of AMT1;2. This concept is further supported by the findings in bzr1-D roots, where the enrichment of 15NH4+ was higher than that in the wild-type. Therefore, in the presence of ammonium, a non-significant increase in NH4+ influx may be related to the down regulation of AMT mRNA levels (Yuan et al., 2007) or AMT protein activity (Lanquar et al., 2009; Yuan et al., 2013) at posttranscriptional or posttranslational levels. Furthermore, increased BZR1 expression levels were also associated with increased ammonium abundance in roots. We observed that all these NH4+ uptake-related traits were affected in these lines with or without additional BR treatment. This observation suggests that BZR1 is a positive regulator of NH4+ uptake under any condition conducive to plant growth. To sum up, BZR1 is the coordination center between NH4+ absorption and general...
growth promotion mediated by BRs. AMT1;2 transcriptional level changes are significantly affected by BZR1 levels, suggesting a BZR1-dependent ammonium uptake pathway in roots. We have done further research on whether BZR1 directly regulates AMT1;2 expression. Nine motifs were identified in the 2.5 kb promoter region of AMT1;2 by promoter sequence analysis. EMSA, CHIP, and transient gene expression tests were used to further study the interaction between AMT1;2 and BZR1. The two BRRE motifs in the promoter have been shown to be sites that bind BZR1 and directly activate AMT1;2 transcription. In addition, although BZR1 can bind both BRRE motifs, the binding strength is different. It is likely that the positions of BRRE motifs in the promoter plays an important role in the regulation of AMT1;2. The comparison of BR- and NH4+ -mediated expression of AMT1;2 in BZR1 RNAi, brz1-D, and wild-type plants indicates that constitutive activation of AMT1;2 expression via BZR1 is independent of external signal stimulation. BRI1 performs a receptor function in BR signaling pathways and actively regulates BR-dependent AMT1;2 expression through its signal reception. In addition, the roots of d61-1, the BRI1 mutant, accumulate less NH4+ than the corresponding wild-type, suggesting a positive role of BRI1 in regulating AMT1;2 expression in roots. The key BR signaling regulators BZR1, the BR receptor BRI1 play the putative role in the regulation of AMT1;2 gene expression. Therefore, clarifying their inter-relationships enhances our understanding of the relationship between BR signaling and the mechanism of AMT regulation. We measured BR- or NH4+ -mediated AMT1;2 gene expression levels and 15NH4+ absorption in three rice lines with different combinations of BZR1 and BRI1 gene expression levels, including the line brz1-D in the d61-1 background and the line br1-D in the BZR1 RNAi background. The activation of BRI1-induced AMT1;2 requires BZR1 involvement, while the process of activation by BZR1 does not require BRI1 activity. Therefore, BZR1 plays a key role in coordinating root uptake of ammonium and BR-dependent plant growth gene expression regulation. Our study further elucidated a relationship between the two key factors BZR1 and BRI1 in the BR signaling pathway associated with regulation of AMT1;2 gene expression and demonstrated that BZR1 was localized downstream of the BRI1 during regulation of AMT1;2 expression. It has been reported that BZR1 act as an integrator or master regulator to regulate plant growth, development, and immunity by directly interacting with key proteins from hormone signaling, stress signaling, cell elongation, flowering,
Brassinosteroids play diverse functions in plant growth and development. In this study, we examined the role of BRs in ammonium uptake in rice. The data indicate that BR signaling activates AMT1;1 and AMT1;2 expression in the presence of the BR receptor BRI1, and the BR signaling transcription factor BZR1 directly activates AMT1;2. The further genetic study revealed that BZR1 activates AMT1;2 expression downstream of BRI1 to improve ammonium uptake in rice. These results indicate that BR signaling positively controls ammonium uptake partially via BZR1-mediated activation of AMT1;2, one of the key AMTs. Our analyses extend the knowledge of the BR-regulating module and its role in the regulation of nitrogen uptake in rice plants.

DATA AVAILABILITY STATEMENT
The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS
SY, YZ, and DY conceived this project. QS and YX provided the experimental design ideas and plant materials. YZ, SY, and DY carried out the experiments and generated the data. QS, YX, and DY contributed to the summary and analysis of the data. QS, YX, YZ, and SY wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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