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Effect of brassinosteroids on ammonium uptake *via* regulation of ammonium transporter and N-metabolism genes in *Arabidopsis*

B.T. ZHAO[1]*, X.F. ZHU[2], J.H. JUNG[3], and Y.H. XUAN[2]*

College of Life Sciences, Shandong Normal University, Jinan, Shandong 250014, P.R. China[1]
College of Plant Protection, Shenyang Agricultural University, Shenyang, Liaoning 110866, P.R. China[2]
Department of Biotech R&D, Amicogen Inc., Jinju 660751, South Korea[3]

Abstract

Several studies have been performed to elucidate the role of brassinosteroids (BRs) in plant growth and development. However, information on the role of BR signaling in nutrient uptake is limited. This study explores the relationship between BRs and *ammonium transporter 1* (*AMT1*) expression in *Arabidopsis* roots. We found that BR treatment reduced the expression of *AMT1* genes and that a BR receptor BRI1 mutant *bri1-5* reversed its BR-repressed expression. Furthermore, the BR signaling transcription factor, BES1, regulates *AMT1* expression in roots. NH_4^+-mediated repression of *AMT1;1*, *AMT1;2,* and *AMT1;3* was suppressed in a gain-of-function *BES1* mutant (*bes1-D*). This mutant was more sensitive to methyl-ammonium and contained a higher ammonium content compared to wild-type plants. However, BES1 failed to bind E-box elements present in the promoter region of the *AMT1* genes. Furthermore, NH_4^+-mediated glutamine synthetase (*GS*) and glutamine oxoglutarate aminotransferase (*GOGAT*) gene expressions were partially inhibited, and GS activity was slightly lower in the *bes1-D* mutant relative to that observed in wild-type En2 roots. NH_4^+-mediated *AMT1* suppressions are known to be caused by N-metabolites rather than NH_4^+ itself, and glutamine application inhibited *AMT1* expression in both En2 and *bes1-D* indicating that BES1 activation inhibited NH_4^+-mediated *GS/GOGAT* induction, which might in turn inhibit *AMT1* repression. In conclusion, the present study demonstrates that BR regulated nitrogen uptake and assimilation *via* the BR signaling pathway.

Additional key words: brassinosteroid signaling transcription factor, glutamate synthase, glutamine synthetase, mutant.

Introduction

Nitrate and ammonium are the major sources of nitrogen in higher plants. NH_4^+ ions accumulate in cells either by direct uptake from the rhizosphere *via* ammonium transporters (AMTs) or by reduction of NO_3^-. The energy cost of reducing nitrate to NH_4^+ consumes 12 - 26 % of photosynthetically-generated reductants. Therefore, using NH_4^+ as nitrogen source conserves a large amount of energy for plants (Bloom 1997, Noctor and Foyer 1998, Patterson *et al.* 2010). Although NH_4^+ is an energetically favorable N source, various plants exhibit toxic symptoms in response to high NH_4^+ concentrations (Britto and Kronzucker 2002). *Arabidopsis* is an NH_4^+-sensitive species, and all *AtAMT1* (*AtAMT1;1*, *AtAMT1;2*, and *AtAMT1;3*) and *AtAMT2;1* gene expressions are suppressed with a high content of NH_4^+ (Sohlenkamp *et al.* 2000, Loque *et al.* 2006). Uptake of N^{15}-labeled NH_4^+ has identified that *AMT1;1* and *AMT1;3* mutations reduce a high-capacity NH_4^+ uptake by 60 - 70 % (Loque *et al.* 2006). Furthermore, the regulatory mechanism underlying this activity has been determined to involve NH_4^+ assimilation products rather than NH_4^+ itself, being responsible for *AMT* expression (Rawat *et al.* 1999). In

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Abbreviations: AMT - ammonium transporter; BES - brassinosteroid signaling transcription factor; BL - brassinolide; BR - brassinosteroid; ChIP - chromatin immunoprecipitation; Col-0, En2, and WS2 - wild-types; GFP - green fluorescent protein; GOGAT - glutamate synthase; GS - glutamine synthetase; MeA - methylammonium; qko - quadruple mutant; qPCR - quantitative PCR.
Acknowledgements: The *bri1-5, bes1-D* and 35S:BES1:GFP seeds were obtained from Wang Laboratory of the Carnegie Institution for Science as a kind gift. The *qko* and *qko+ATM1;1* seeds were obtained from Dr. Nicolaus Von Wirén's group at Leibniz Institute for Plant Genetics and Crop Plant Research. This work was supported by grants from the Wenzhou City public welfare technology projects (N20150021) and the Shenyang Agricultural University.
* Corresponding authors; fax: (+86) 24 88342065, e-mail: yuanhu115@naver.com; fax: (+86) 531 86183421, e-mail: tengda888666@sina.com

Arabidopsis roots, *GS/GOGAT* genes, encoding glutamine synthetase and glutamine oxoglutarate aminotransferase also known as glutamate synthase, are upregulated following re-supply of NH_4^+ (Konishi *et al.* 2014).

Phytohormones play important roles in NH_4^+-related plant growth and stress responses. Auxin-resistant *aux1*, *axr1*, and *axr2* mutants are insensitive to NH_4^+-mediated root growth inhibition (Cao *et al.* 1993). Activation of abscisic acid signaling reduces NH_4^+-induced stress in the plastid *AMOS1*/*EGY1* mutant (Li *et al.* 2012). NH_4^+ applied to shoots causes the auxin-influx carrier AUX1 to inhibit lateral-root emergence (Li *et al.* 2011). Ethylene production in shoots is associated with NH_4^+-mediated lateral-root inhibition (Li *et al.* 2013). The mechanisms underlying hormonal regulation of NH_4^+ stress are largely unknown. Systemic approaches have been used to explore *Arabidopsis* gene expression in response to brassinosteroids (BRs) as well as in a gain-of-function of the BR signaling transcription factor (BES1) mutant, *bes1-D*. These studies have shown that *AtAMT1;1* is upregulated by the activation of BR signaling (Goda *et al.* 2004, Yu *et al.* 2011).

Brasinosteroid perception by the brassinosteroid insensitive 1 (BRI1) receptor leads to dissociation of BRI1 kinase inhibitor 1 (BKI1) and association of BRI1-associated receptor kinase 1 (BAK1). Activation of BRI1-BAK1 eventually results in dephosphorylation and activation of brassinazole-resistant 1 (BZR1) and BRI1-EMS-suppressor 1 (BES1), two transcription factors that regulate target gene expression in response to BRs (Li and Chory 1997, Li *et al.* 2002, Nam and Li 2002, Kim and Wang 2010, Yang *et al.* 2011). Protein stability is significantly elevated in *bes1-D* and *bzr1-D* gain-of-function *BES1* and *BZR1* mutants, in which a single proline within the domain enriched in proline, glutamic acid, serine, and threonine (PEST) is substituted by leucine, which in turn rescues the phenotype of the *bri1* BR-receptor mutant (Wang *et al.* 2002, Yin *et al.* 2002).

In the present study, we analyzed the effects of BR on *AMT1* expression in *Arabidopsis* using mutants of a BR-receptor and a BR-signal related E-box-binding transcription factor. The expression pattern of the *AMT1* family was examined in *bri1-5*. *Arabidopsis bes1-D* was utilized to determine the effect of BR signal-related transcription factors on *AMT1* and *GS/GOGAT* gene expressions in roots. Cellular ammonium content, sensitivity to methylammonium (MeA), and root growth were analyzed to verify the involvement of BR in NH_4^+-mediated root development in *Arabidopsis*. Furthermore, feedback regulation of *AMT1* expression via metabolites was analyzed in *bes1-D*. This is the first study to investigate the hypothesis that ammonium uptake and assimilation are regulated by BR signaling.

Materials and methods

Plants and growth conditions. To examine NH_4^+-mediated *AMT1* gene expression, surface-sterilized *Arabidopsis thaliana* L. seeds of the genotype Col-0 (control for *35S:BES1:GFP*), W2 (control for *bri1-5*), and E2 (control for *bes1-D*) used as wild types, *35S:BES1-GFP*-expressing transgenic plants (obtained from Wang Laboratory of the Carnegie Institution for Science), and mutants *bril-5*, *bes1-D*, *qko* (obtained from the Leibniz Institute for Plant Genetics and Crop Plant Research) were sown in a half strength Murashige and Skoog (0.5× MS) medium. After two days of stratification at 4 °C, the seedlings were incubated at a temperature of 22 °C, a 16-h photoperiod, an irradiance of 80 μmol m^{-2} s^{-1}, and a relative humidity of 40 % for 7 d. The seedlings were then transferred to N-free 0.5× MS medium for 3 d prior to ammonium treatment. Roots were sampled at 0 and 6 h after addition of 2.5 mM $(NH_4)_2SO_4$. For BR treatment, the seeds germinated and grown for 10 d in a modified-MS medium containing 10 mM KNO_3 as sole N source were transferred to the same medium containing 100 nM brassinolide (BL), and seedling roots were sampled at 0 and 3 h after the BL treatment. For NH_4^+-uptake experiments, 5 mM MeA was added to a modified-MS media with 1 mM NH_4NO_3 as N source. The seeds were sown in an MeA-containing medium and grown for 7 d. For testing NH_4^+ effects on primary root growth, the seeds were sown and grown for 12 d in the modified 0.5× MS with 0.5 mM KNO_3 as sole N source or in a modified 0.5× MS containing 0.5 mM KNO_3 and 0.5, 1, 2, or 5 mM NH_4Cl. All plant-growth media were buffered with 1 mM 2-(*N*-morpholino) ethanesulfonic acid (MES; pH 5.8). To analyze BL effects on NH_4^+ uptake, the seeds germinated and grown for 10 d in a modified-MS medium containing 10 mM KNO_3 as sole N source were transferred to an MS medium containing 0 and 100 nM BL, and seedling roots were sampled after 0, 3, 6, and 9 h.

RNA extraction and real time qPCR analysis. The total cellular RNA was isolated with an *RNeasy* plant mini kit (*Qiagen*, USA) or a *Trizol* reagent (*Takara*, Dalian, China) and subsequently treated with *RQ-RNase-free DNase* (*Promega*, Madison, WI, USA) to eliminate genomic DNA contamination. For cDNA synthesis, a reverse transcriptase *RNaseH* (*Toyobo*, http://www.toyobo-global.com/) or *GoScript* reverse transcription kit was used following the manufacturer's instructions (*Promega*). Real time qPCR products were quantified using an *Illumina Research Quantity v. Eco 3.0* (*Illumina*, San Diego, CA, USA), and values were normalized

against *actin* expression in the same samples. The primers used for real time qPCR analysis are presented in Table 1 Suppl.

Chromatin immunoprecipitation assay. Approximately 2 g of 2-d-old Col-0 and *35S:BES1-GFP*-expressing seedlings were prepared for chromatin immune-precipitation (ChIP) assay. Pre-absorption with a pre-immune serum was performed before immune-precipitation with a green fluorescent protein (GFP) monoclonal antibody (*Clontech*, USA). The immune-precipitates were analyzed by ChIP-PCR as described above. Each input DNA level was used as control to normalize the immunoprecipitate ratio (Je *et al.* 2010). The primers used for ChIP-PCR analysis are listed in Table 1 Suppl.

Determination of NH_4^+ content, chlorophyll content, and glutamine synthetase activity: For testing NH_4^+ content, 7-d-old plant roots were sampled and weighted before freezing in liquid nitrogen. The root tissue was then ground to powder and enzymatic determination of NH_4^+ content in roots was performed according to Oliveira *et al.* (2002) using an *F-kit* (*Roche*, USA) and following the manufacturer's instructions.

Whole shoots of 7-d-old En2 and *bes1-D* plants with or without a 5 mM methyl-ammonium application was sampled and weighed before keeping in 100 % EtOH with gentle shaking at room temperature. After shoot tissue became completely white, the chlorophyll content in the EtOH solution was determined by a UV spectro-photometer as previously described (Lichtenthaler 1987).

For GS assay, shoots and seminal roots frozen in liquid nitrogen were ground and dissolved in 1 cm^3 of cold 50 mM imidazole-HCl (pH 7.2) containing 0.5 mM EDTA, 1 mM dithiothreitol, 10 mM $MgSO_4$, 10 % (v/v) glycerol, and 1 mM phenylmethane sulfonyl fluoride. Following centrifugation at 15 000 *g* for 20 min, hydroxylamine and ATP were added to the supernatant and kept at 37 °C. After 30 min 0.2 M trichloroacetic acid, 0.37 M $FeCl_3$, and 0.6 M HCl) was added and centrifuged at 5 000 g for 10 min before measuring absorbance at 540 nm. The activity of GS was determined as described by Mack (1995).

Statistical analysis: Statistical calculations were conducted using *Prism 5* (*GraphPad*, San Diego, CA, USA). All data were expressed as means ± SEs. Comparison between two groups was performed by using the *t*-test.

Results

To investigate the effect of *BRI1* on *AtAMT1* expression in *Arabidopsis* roots, the plants were grown on a modified-MS medium in which nitrate was the sole N source because NH_4^+ significantly suppresses *AtAMT1* expression in *Arabidopsis* roots. Wild-type plants (WS2) and *bri1-5*, a weak *BRI1* mutant, were grown for 10 d and then transferred to the same medium containing 100 nM BL. After 3 h, roots were examined for *AtAMT1;1*, *AtAMT1;2*, and *AtAMT1;3* expressions by real time qPCR. The results show that the expressions of all three *AtAMT1* genes were inhibited by BR, but this repression was fully or partially suppressed in *bri1-5* mutant roots (Fig. 1). *BR6OX2* encodes a BR-biosynthesis enzyme that converts 6-deoxocastasterone into castasterone; *BR6OX2* expression is known to be inhibited upon BR signaling activation (Oh *et al.* 2012). *BR6OX2* expression was higher in *bri1-5* than in WS2 wild-type roots, which could be due to a negative feedback of the BRI1 receptor in regulating BR-biosynthesis gene expression (Fig. 1*D*). Furthermore, BL effects on ammonium uptake were analyzed in BL-treated and untreated roots. The results indicate that the BL treatment enhanced cellular ammonium content after 3 and 6 h of the treatment (Fig. 1 Suppl.). The results indicate that BR signaling activation accumulated more cellular NH_4^+, and suppression of BR-mediated *AtATM1*-repression was inhibited in *BRI1* mutant *bri1-5*, suggesting that BR-mediated *AtATM1*-repression required BRI1 activity in *Arabidopsis* roots

To further elucidate the BR-signaling pathway involved in BR-dependent *AtAMT1* expression in *Arabidopsis* roots, the BRI1 downstream regulator BES1 was examined for effects on *AtAMT1* gene expressions with or without NH_4^+ supplementation. In addition, microarray data show that *AtAMT1;1* expression was higher in the gain-of-function *bes1-D* mutant than in its corresponding wild-type En2 (Yu *et al.* 2011). The *bes1-D* and En2 plants were grown in the 0.5× MS for 7 d, and then transferred to the N-free 0.5× MS medium for an additional 3 d. The plants were then transferred to the modified-MS medium with 2.5 mM $(NH_4)_2SO_4$ as sole N source. Ribonucleic acids were extracted from whole roots that were sampled 0 and 6 h after the transfer. NH_4^+-mediated *AtAMT1;1*, *AtAMT1;2*, and *AtAMT1;3* expression was measured by real time qPCR. *AtAMT1;1*, *AtAMT1;2*, and *AtAMT1;3* expression was suppressed in the wild-type plants after exposure to the NH_4^+ treatment (Figs. 2*A-C* and Fig. 1 Suppl.). However, *AtAMT1;1* became insensitive to NH_4^+ in *bes1-D* mutant roots; *AtAMT1;1* expressions were the same in *bes1-D* roots with or without NH_4^+. In contrast, *AtAMT1;3* expression was reduced in *bes1-D* and was not suppressed by NH_4^+, whereas *AtAMT1;2* expression was suppressed in *bes1-D* and by NH_4^+ (Fig. 2*A-C*). Therefore, *bes1-D* disrupted NH_4^+-mediated *AtAMT1;1*, *AtAMT1;2*, and *AtAMT1;3* repression. Furthermore, NH_4^+-mediated *BES1*

expression was analyzed, and *BES1* transcription was not altered by the NH_4^+ treatment (Fig. 2*D*).

NH_4^+-mediated repression of *AtAMT1;1*, *AtAMT1;2*, and *AtAMT1;3* expression was suppressed in *bes1-D*. Therefore, the possibility that BES1 directly regulated *AtAMT1* expression by binding to its promoter was examined. Promoter-sequence analysis identified several E-box elements (CANNTG) in the 1.5-kb region of the *AtAMT1* promoters (Fig. 2 Suppl.). To test the binding affinity of BES1 to the E-box elements *in planta*, a ChIP assay was performed using 2-d-old Col-0 and *35S:BES1-GFP*-expressing transgenic plants. Relative immunoprecipitation amounts were measured with ChIP-PCR using primer pairs amplifying P1-P6 regions that encompassed the E-box motifs (Fig. 2 Suppl.). Six primer sets from the *BES1-GFP* line did not produce PCR products that would be different from those of the Col-0 plants (Fig. 2 Suppl.). The ChIP assay failed to indicate that BES1 indirectly regulated NH_4^+-responsive *AtAMT1* genes.

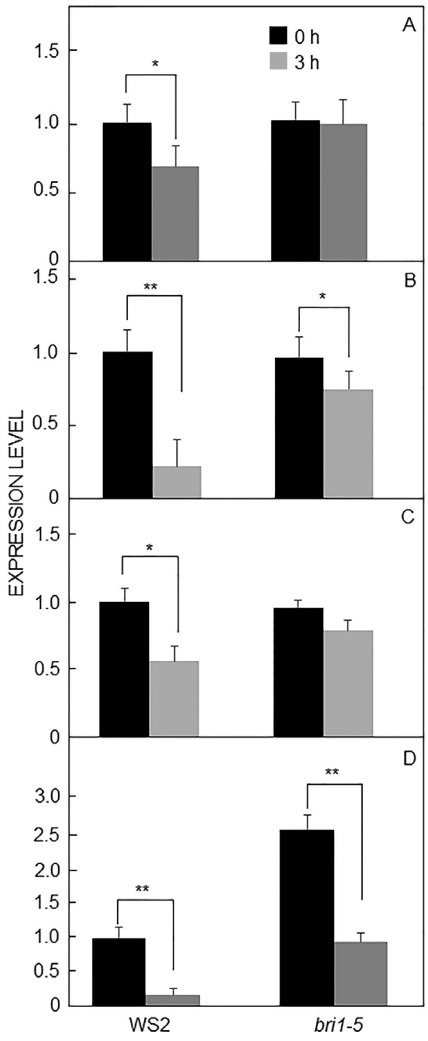

Fig. 1. Brassinosteroid (BR)-dependent *AtAMT1* and *BR6OX2* expression patterns in a wild-type (WS2) and *bri1-5* roots. Seedlings were grown for 7 d and treated with 100 nM brassinolide for 3 h. BR-mediated relative expression was analyzed by real time qPCR: *A* - *AtAMT1;1*, *B* - *AtAMT1;2*, *C* - *AtAMT1;3*, *D* - *BR6OX2*. *Actin* was used as control. The expressions of *AtAMT1;1*, *AtAMT1;2*, *AtAMT1;3*, and *BR6OX2* in WS2 before brassinolide treatment (0 h) were defined as "1". Means ± SEs (n = 3). *,** - significant differences at $P < 0.05$ and $P < 0.01$, respectively.

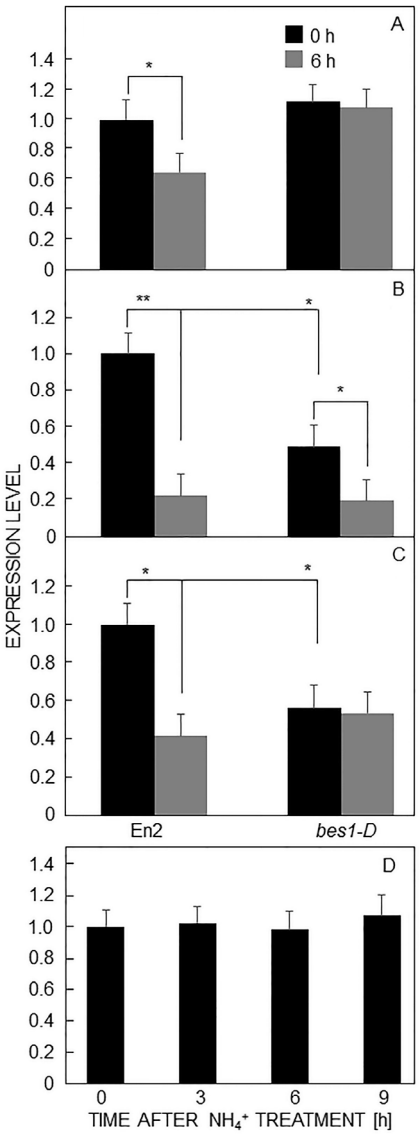

Fig. 2. NH_4^+-mediated *AtAMT1* expression in selected mutants. *AtAMT1;1* (*A*), *AtAMT1;2* (*B*), and *AtAMT1;3* (*C*) expression patterns in a wild-type (En2) and *bes1-D* plant roots before or 6 h after 2.5 mM $(NH_4)_2SO_4$ treatment measured by real time qPCR. The expressions of *AtAMT1;1*, *AtAMT1;2*, and *AtAMT1;3* in En2 before NH_4^+ treatment (0 h) were defined as "1". Means ± SEs (n = 3). *,** - significant differences at $P < 0.05$ and $P < 0.01$, respectively. *D* - Time-dependent *BES1* relative expression was analyzed in *Arabidopsis thaliana* Col-0 roots 0, 3, 6, and 9 h after NH_4Cl treatment. Plants were grown in a 0.5× MS for 7 d, deprived of N for 3 d, and then transferred to a medium containing 2.5 mM $(NH_4)_2SO_4$. *Actin* was used as reference gene. The expression level of *BES1* in Col-0 before NH_4^+ treatment (0 h) was defined as "1". Means ± SEs (n = 3).

To estimate the effect of *BES1* on NH_4^+ uptake and NH_4^+-mediated growth, cellular NH_4^+ content, sensitivity to MeA, a toxic ammonium analog, and root growth rate were measured. The content of NH_4^+ was measured in En2, *bes1-D*, an *AtAMT* quadruple mutant (*qko*), and *qko+AtAMT1;1*, in which *AtATM1;1* was reintroduced into *qko* (Yuan *et al.* 2007). Since *amt1;1* and *amt1;2* are of a Col-0 background, whereas *amt1;2* has a WS2 background (Loque *et al.* 2006; Yuan *et al.* 2007), *qko+AtAMT1;1* was utilized as control of *qko* to avoid the effects of chromosomal contamination. The plants were grown in the 0.5× MS for 7 d, and whole roots were sampled to measure NH_4^+ content. The *bes1-D* and *qko+AtAMT1;1* accumulated higher amounts of NH_4^+ compared to the wild-type and *qko* plants, respectively (Fig. 3*A*). As expected, NH_4^+ content was lower in *qko* than in *qko+AtAMT1;1*. To estimate sensitivity to MeA, chlorophyll content was compared in the En2 and *bes1-D* seedlings grown for 7 d in the presence of MeA. The plants were grown in a medium containing 1 mM NH_4NO_3 with or without 5 mM MeA (Figs. 3*B* and Fig. 3 Suppl.). Without MeA, *bes1-D* accumulated approximately 15 % less chlorophyll than En2. However, in the presence of 5 mM MeA, *bes1-D* chlorophyll content was approximately 50 % lower than that of En2. Severe chlorosis was observed in the *bes1-D* seedlings supplemented with 5 mM MeA (Figs. 3 Suppl.).

The effect of *BES1* on root growth in the presence of NH_4 was also examined. Primary root lengths were measured in the plants grown for 12 d in the modified

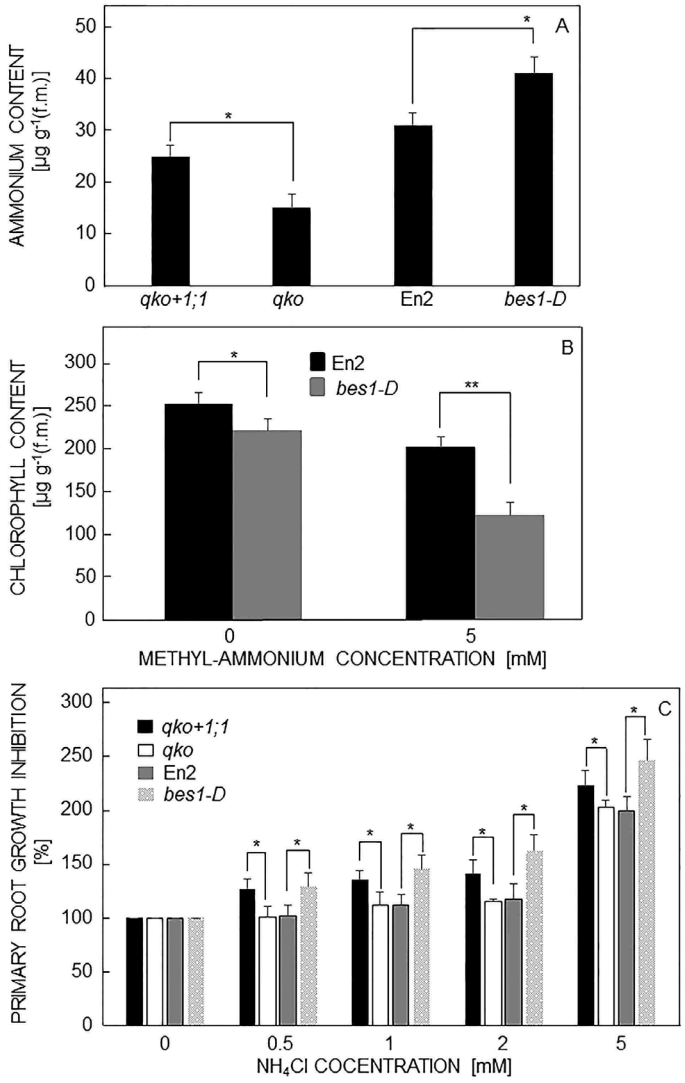

Fig. 3. Cellular NH_4^+ content, methylammonium (MeA) sensitivities, and root growth rates of selected mutants. *A* - NH_4^+ content in roots of plants grown in a 0.5× MS for 7 d. *B* - Chlorophyll content in En2 and *bes1-D* shoots was measured after growth in a modified MS medium containing 1 mM NH_4NO_3 and indicated concentration of MeA for 7 d; *C* - Growth rates of primary roots of 12-d-old plants grown in a modified MS medium containing 0.5 mM KNO_3 as sole N source before adding the indicated concentrations of NH_4Cl and treated for 12 d. Means ± SEs (n >10 plants); *, ** - significant differences at $P < 0.05$ and $P < 0.01$, respectively.

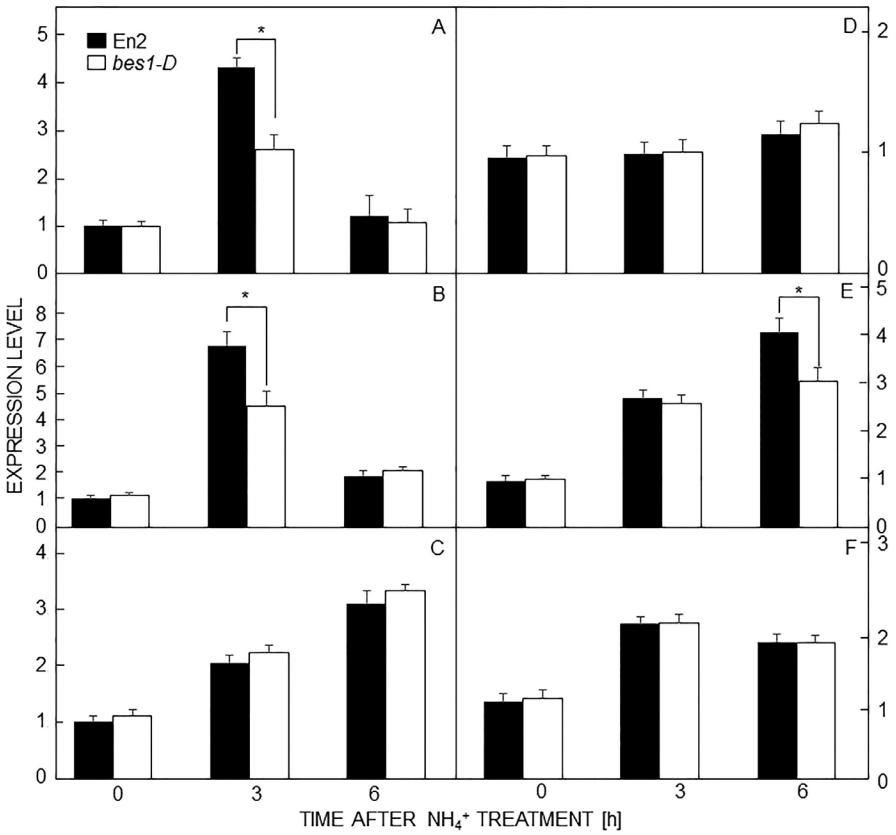

Fig. 4. NH_4^+-mediated expression patterns of glutamate synthase and glutamine synthetase genes in En2 and *bes1-D* roots analyzed by real time qPCR. Plants were supplied with a 0.5× MS medium for 10 d, then deprived of N for 3 d prior to transfer to 2.5 mM $(NH_4)_2SO_4$. *A - Ferredoxin-dependent glutamate synthase* (AT5G04140); *B - Glutamate synthase 2* (AT2G41220); *C - NADH-dependent glutamate synthase 1* (AT5G53460); *D - Glutamine synthetase 1;2* (AT1G66200); *E - Glutamine synthetase 2* (AT5G35630); *F - Folylpolyglutamate synthetase 2* (AT3G10160). The expressions in En2 before NH_4^+ treatment (0 h) were defined as "1". *Actin* was used as internal control. Means ± SEs of three replicates. * - significant differences at $P < 0.05$.

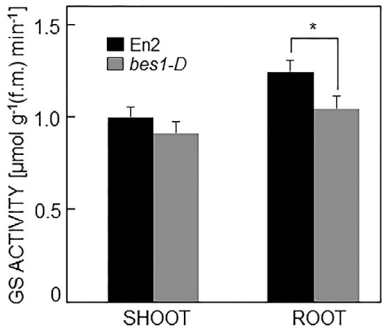

Fig. 5 Glutamine synthetase (GS) activity in shoots and seminal roots of 13-d-old wild-type (En2) and *bes1-D* plants. Means ± SEs of three replicates, and experiments were repeated three times. * - significant differences at $P < 0.05$.

0.5× MS with 0.5 mM KNO_3 as sole N source or in the modified 0.5× MS with 0.5 mM KNO_3 and 0.5, 1, 2, or 5 mM NH_4. Because *bes1-D* roots were shorter than those of En2 when grown without NH_4Cl, the effect of NH_4^+ on primary roots was measured as the percentage of inhibition (Figs. 3*C*, Fig. 3 Suppl.). Compared to that of En2, *bes1-D* root growth was significantly affected by 5 mM NH_4Cl. As expected, *qko+AtAMT1;1* exhibited a significant primary root growth inhibition at high NH_4^+ concentrations compared to that in *qko* (Fig. 3*C*). These findings indicate that *bes1-D* roots were more sensitive to NH_4^+ than En2, whereas *qko* roots were obviously less sensitive to high NH_4^+ concentrations compared to *qko+AtAMT1;1*. NH_4^+-mediated repression of *AMT1* is caused by the NH_4^+ assimilation products instead of NH_4^+ itself being responsible for the expression (Rawat *et al.* 1999). Furthermore, NH_4^+ assimilation-related enzyme expressions were analyzed. In *Arabidopsis* roots, *GS/GOGAT* genes have been reported to be induced following NH_4^+ re-supply (Konishi *et al*. 2014), and the six genes, shown in Fig. 4, that encode GS or GOGAT are differentially expressed in *bes1-D* compared to En2 (Yu *et al.* 2011). The *bes1-D* and its corresponding wild-type En2 were grown in the 0.5× MS for 7 d and then moved onto the N-free medium for additional 3 d. The plants were then transferred to the modified MS medium in which 2.5 mM $(NH_4)_2SO_4$ was used as sole N source. Ribonucleic acid extracted from whole roots was sampled at 0, 3, and 6 h after the transfer. NH_4^+-mediated

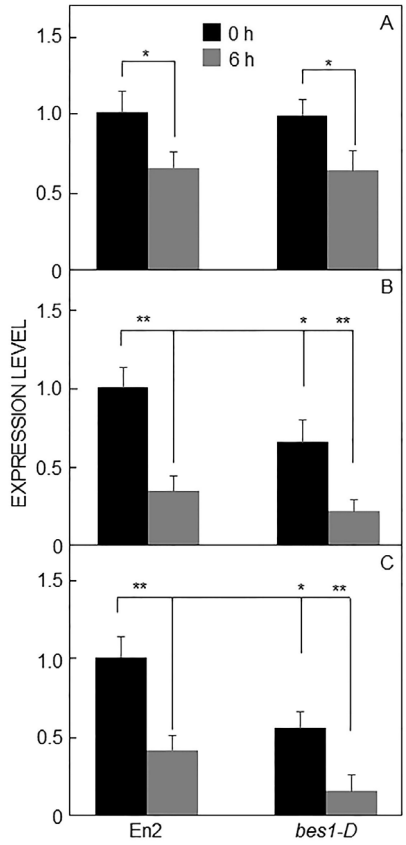

Fig. 6 Glutamine-mediated expression patterns of *AtAMT1;1* (*A*), *AtAMT1;2* (*B*), and *AtAMT1;3* (*C*) analyzed by real time qPCR in En2 and *bes1-D* roots of plants grown in a 0.5× MS for 10 d, then deprived of N for 3 d prior to transfer to 5 mM L-glutamine. *Actin* was used as reference gene. The expressions in En2 before glutamine treatment (0 h) were defined as "1". Means ± SEs of three replicates. Experiments were repeated at least three times. *, ** significant differences at $P < 0.05$ and $P < 0.01$, respectively.

expressions of *GS* (*GS1;2*, *GS2*, and *folylpolyglutamate synthetase 2*) and *GOGAT* (*ferredoxin-dependent glutamate synthase*, *glutamate synthase 2* and *NADH-dependent glutamate synthase 1*) genes, which have been earlier reported to have changed in *bes1-D*, were measured by real time qPCR. Except for *GS1;2*, all of the genes tested were induced upon the NH_4^+ treatment in both En2 roots and *bes1-D* roots (Fig. 4). However, induction kinetics of *GS2*, *ferredoxin-dependent glutamate synthase*, and *glutamate synthase 2* genes were lower in *bes1-D* compared to En2.

Because *GS* gene expressions were obviously lower in *bes1-D* roots, GS activity was further measured in En2 and *bes1-D* plant shoots and roots. The plants were grown on the modified MS medium with 10 mM KNO_3 as sole N source for 10 d and then transferred to the modified MS medium with 2.5 mM $(NH_4)_2SO_4$ for another 3 d. The results show that GS activity was similar in En2 and *bes1-D* shoots, whereas it was lower in *bes1-D* roots than in En2 roots (Fig. 5) implying that BES1-induced regulation of GS activity might be *via* inhibition of NH_4^+-mediated induction of *GS* genes.

NH_4^+ was incorporated into the amide group of glutamine after its assimilation into cells. Glutamine and NH_4^+ showed the same effects on *AMT1;1* expression although NH_4^+ together with methionine sulfoximine, an inhibitor of NH_4^+ assimilation, inhibited NH_4^+-repression of *AMT1;1*, suggesting that NH_4^+ metabolites rather than NH_4^+ itself repress *AMT1;1* expression in *Arabidopsis* roots (Rawat *et al.* 1999). To understand whether the effect of *bes1-D* on *AMT1* expression in roots mainly relied on cellular amino acid content, the En2 and *bes1-D* plants were grown on the MS medium for 7 d and then were transferred to the N-free medium and grown for another 3 d prior to treatment with L-glutamine. Real time qPCR shows that *AMT1;1-1;3* had similar suppression kinetics in both En2 roots and *bes1-D* roots upon glutamine application (Fig. 6). These findings suggest that BES1 was involved in NH_4^+-mediated circuits which regulated *AMT1* expression most likely *via* regulation of N-metabolism.

Discussion

The primary objective of the present study was to understand BR-mediated *AMT1* expression in *Arabidopsis*. In *Arabidopsis*, the genome-wide expression analysis with BR-treated wild-type plants or *bes1-D* reveals that activation of BR signaling upregulated *AtAMT1;1* (Goda *et al.* 2004, Yu *et al.* 2011). The present study explored the possibility that *BRI1*-mediated BR signaling could influence *AMT1* expression and NH_4^+-responsive root growth in *Arabidopsis*. To achieve this objective, the mutants of *BRI1* and BR-signaling activator *BES1* were utilized.

The study shows that *AtAMT1* expression in roots was regulated by *BRI1*-mediated signaling in plants. In *Arabidopsis*, BR-mediated changes in *AtAMT1* expression required BRI1 BR-receptor activity. *AtAMT1* in *Arabidopsis* roots was negatively regulated by BR. The *bes1-D* exhibits constitutive BR response and rescues the *bri1* mutant phenotype (Yin *et al.* 2002). Our analyses show that *AtAMT1* expression was inhibited by exogenously supplied BR in *bes1-D* mutant roots. The BR treatment enhanced cellular NH_4^+ content and the *bes1-D* plants accumulated more NH_4^+ in cells than the wild-type suggesting that activation of BR signaling increased NH_4^+ uptake, which might in turn repress *AtAMT1* gene expression. The extensive analysis of *bes1-D* shows that BR could affect NH_4^+ uptake-related root growth. We were unable to identify the binding affinity of BES1 to the *AtAMT1* promoters; therefore, the

regulatory mechanism whereby BES1 influences *AtAMT1* expression is unclear. NH_4^+ re-supply suppressed *AMT1* expression, whereas NH_4^+-mediated *AMT1* repression was inhibited in *bes1-D*. Interestingly, NH_4^+-mediated *GS/GOGAT* gene expression patterns in En2 and *bes1-D* reveal that *bes1-D* partially inhibited NH_4^+-dependent *GS/GOGAT* induction (Fig. 4). Furthermore, GS activity was lower in *bes1-D* roots compared to En2 roots, and re-supply of L-glutamine repressed *AMT1* expression in both En2 and *bes1-D* suggesting that the lower metabolite content in *bes1-D* roots might result in inhibition of NH_4^+-mediated N metabolism genes in *bes1-D*. *BES1* transcription was not altered by the NH_4^+ treatment. BES1 phosphorylation has been reported to trigger BES1 degradation, and BR treatment attenuates BES1 phosphorylation (Yin *et al.* 2002). Therefore, investigating the effect of NH_4^+ on BES1 phosphorylation may be an interesting topic in future research studies.

Another key BR signaling transcription factor BZR1 has been reported to directly target the general control non-repressible (GCN) in *Arabidopsis*, which is suggestive of a potential role of GCN in BR-dependent regulation of chloroplast development and N metabolism (Sun *et al.* 2010). Therefore, *BZR1* and *BES1* might be involved in different pathways of BR-mediated N-metabolism or NH_4^+ uptake. These observations suggest that distinct N-uptake and assimilation genes are subjected to different BR-mediated regulatory circuits. These results also indicate that a certain regulatory machinery occurs between hormone and nutrient signaling in at least N-metabolism in plants. Further genetic experiments are required to isolate regulators of N-metabolism, which are modulated by BR.

References

Bloom, A.J.: Nitrogen as a limiting factor: crop acquisition of ammonium and nitrate. - In: Jackson, L.E. (ed.): Ecology in Agriculture. Pp. 145-172, Academic Press, San Diego 1997.

Britto, D.T., Kronzucker, H.J.: $NH4^+$ toxicity in higher plants: a critical review. - J. Plant Physiol. **159**: 567-584, 2002.

Cao, Y., Glass, A.D., Crawford, N.M.: Ammonium inhibition of *Arabidopsis* root growth can be reversed by potassium and by auxin resistance mutations *aux1*, axr1, and *axr2*. - Plant. Physiol. **102**: 983-989, 1993.

Goda, H., Sawa, S., Asami, T., Fujioka, S., Shimada, Y., Yoshida, S.: Comprehensive comparison of auxin-regulated and brassinosteroid-regulated genes in *Arabidopsis*. - Plant. Physiol. **134**: 1555-1573, 2004.

Je, B.I., Piao, H.L., Park, S.J., Park, S.H., Kim, C.M., Xuan, Y.H., Park, S.H., Huang, J., Do, C.Y., An, G., Wong, H.L., Fujioka, S., Kim, M.C., Shimamoto, K., Han, C.D.: RAV-Like1 maintains brassinosteroid homeostasis *via* the coordinated activation of *BRI1* and biosynthetic genes in rice. - Plant Cell **22**: 1777-1791, 2010.

Kim, T.W., Wang, Z.Y.: Brassinosteroid signal transduction from receptor kinases to transcription factors. - Annu. Rev Plant Biol. **61**: 681-704, 2010.

Konishi, N., Ishiyama, K., Matsuoka, K., Maru, I., Hayakawa, T., Yamaya, T., Kojima, S.: NADH-dependent glutamate synthase plays a crucial role in assimilating ammonium in the *Arabidopsis* root. - Physiol. Plant. **152**:138-151, 2014.

Li, B., Li, Q., Su, Y., Chen, H., Xiong, L., Mi, G., Kronzucker, H.J., Shi, W.: Shoot-supplied ammonium targets the root auxin influx carrier AUX1 and inhibits lateral root emergence in *Arabidopsis*. - Plant. Cell Environ. **34**: 933-946, 2011.

Li, B., Li, Q., Xiong, L., Kronzucker, H.J., Kramer, U., Shi, W.: *Arabidopsis* plastid AMOS1/EGY1 integrates abscisic acid signaling to regulate global gene expression response to ammonium stress. - Plant. Physiol. **160**: 2040-2051, 2012.

Li, G., Li, B., Dong, G., Feng, X., Kronzucker, H.J., Shi, W.: Ammonium-induced shoot ethylene production is associated with the inhibition of lateral root formation in *Arabidopsis*. - J. exp Bot. **64**: 1413-1425, 2013.

Li, J., Chory, J.: A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. - Cell **90**: 929-938, 1997.

Li, J., Wen, J., Lease, K.A., Doke, J.T., Tax, F.E., Walker, J.C.: BAK1, an Arabidopsis LRR receptor-like protein kinase, interacts with BRI1 and modulates brassinosteroid signaling. - Cell **110**: 213-222, 2002.

Lichtenthaler, H.K.: Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. - Methods. Enzymol. **148**: 350-382, 1987.

Loque, D., Yuan, L., Kojima, S., Gojon, A., Wirth, J., Gazzarrini, S., Ishiyama, K., Takahashi, H., Von Wiren, N.: Additive contribution of AMT1;1 and AMT1;3 to high-affinity ammonium uptake across the plasma membrane of nitrogen-deficient *Arabidopsis* roots. - Plant J. **48**: 522-534, 2006.

Mack, G.: Organ-specific changes in the activity and subunit composition of glutamine-synthetase isoforms of barley (*Hordeum vulgare* L.) after growth on different levels of NH_4^+. - Planta **196**: 231-238, 1995.

Nam, K.H., Li, J.: BRI1/BAK1, a receptor kinase pair mediating brassinosteroid signaling. - Cell **110**: 203-212, 2002.

Noctor, G., Foyer, C.H.: A re-evaluation of the ATP:NADPH budget during C3 photosynthesis: a contribution from nitrate assimilation and its associated respiratory activity? - J. exp Bot. **49**: 1895-1908, 1998.

Oh, E., Zhu, J.Y., Wang, Z.Y.: Interaction between BZR1 and PIF4 integrates brassinosteroid and environmental responses. - Nat. cell. Biol. **14**: 802-809, 2012.

Oliveira, I.C., Brears, T., Knight, T.J., Clark, A., Coruzzi, G.M.: Overexpression of cytosolic glutamine synthetase. Relation to nitrogen, light, and photorespiration. - Plant. Physiol. **129**: 1170-1180, 2002.

Patterson, K., Cakmak, T., Cooper, A., Lager, I., Rasmusson, A.G., Escobar, M.A.: Distinct signalling pathways and transcriptome response signatures differentiate ammonium- and nitrate-supplied plants. - Plant Cell Environ. **33**: 1486-1501, 2010.

Rawat, S.R., Silim, S.N., Kronzucker, H.J., Siddiqi, M.Y., Glass, A.D.: Atamt1 gene expression and NH_4^+ uptake in roots of *Arabidopsis thaliana*: evidence for regulation by

root glutamine levels. - Plant J. **19**: 143-152, 1999.

Sohlenkamp, C., Shelden, M., Howitt, S., Udvardi, M.: Characterization of *Arabidopsis* AtAMT2, a novel ammonium transporter in plants. - FEBS. Lett. **467**: 273-278, 2000.

Sun, Y., Fan, X.Y., Cao, D.M., Tang, W., He, K., Zhu, J.Y., He, J.X., Bai, M.Y., Zhu, S., Oh, E., Patil, S., Kim, T.W., Ji, H., Wong, W.H., Rhee, S.Y., Wang, Z.Y.: Integration of brassinosteroid signal transduction with the transcription network for plant growth regulation in *Arabidopsis*. - Dev. Cell. **19**: 765-777, 2010.

Wang, Z.Y., Nakano, T., Gendron, J., He, J., Chen, M., Vafeados, D., Yang, Y., Fujioka, S., Yoshida, S., Asami, T., Chory, J.: Nuclear-localized BZR1 mediates brassinosteroid-induced growth and feedback suppression of brassinosteroid biosynthesis. - Dev. Cell **2**: 505-513, 2002.

Yang, C.J., Zhang, C., Lu, Y.N., Jin, J.Q., Wang, X.L.: The mechanisms of brassinosteroids' action: from signal transduction to plant development. - Mol. Plant **4**: 588-600, 2011.

Yin, Y., Wang, Z.Y., Mora-Garcia, S., Li, J., Yoshida, S., Asami, T., Chory, J.: BES1 accumulates in the nucleus in response to brassinosteroids to regulate gene expression and promote stem elongation. - Cell **109**: 181-191, 2002.

Yu, X., Li, L., Zola, J., Aluru, M., Ye, H., Foudree, A., Guo, H., Anderson, S., Aluru, S., Liu, P., Rodermel, S., Yin, Y.: A brassinosteroid transcriptional network revealed by genome-wide identification of BES1 target genes in *Arabidopsis thaliana*. - Plant J. **65**: 634-646, 2011.

Yuan, L., Loque, D., Kojima, S., Rauch, S., Ishiyama, K., Inoue, E., Takahashi, H., von Wiren, N.: The organization of high-affinity ammonium uptake in *Arabidopsis* roots depends on the spatial arrangement and biochemical properties of AMT1-type transporters. - Plant Cell **19**: 2636-2652, 2007.