Ammonium uptake capacity and response of cytosolic glutamine synthetase 1;2 to ammonium supply are key factors for the adaptation of ammonium nutrition in Arabidopsis thaliana

doi: 10.1080/00380768.2017.1314178

Ammonium uptake capacity and response of cytosolic glutamine synthetase 1;2 to ammonium supply are key factors for the adaptation of ammonium nutrition in *Arabidopsis thaliana*

Plant requires nitrogen for the growth, and it use nitrate and ammonium from the environment. Plant suffers from the toxicity when excess ammonium is supplied as a sole nitrogen, although it could be a good nitrogen source for plant growth. We hypothesized that the different responses of ecotypes to ammonium nutrient could partly account for the adaptation of Arabidopsis to an ammonium environment. The purpose of this study is to understand the different responses of ecotypes in ammonium environment. The growth of Arabidopsis thaliana ecotypes, Columbia was compared to those of *Arabidopsis thaliana* ecotypes, Landsberg *erecta* in ammonium nutrient. The ratio of shoot dry weight to root dry weight was compared to evaluate the adaptation of two ecotypes. The shoot:root ratio of Landsberg was significantly higher than that of Columbia. T-DNA insertion in cytosolic glutamine synthetase 1;2, one of the essential ammonium assimilatory enzymes, led a decrease of shoot:root ratio. We also measured the isotope labeled ammonium uptake and the expression levels of ammonium transporter genes, and also the expression of ammonium assimilatory genes, glutamine synthetase genes and glutamate synthase genes, in roots after ammonium re-supply using real-time polymerase chain reaction analysis. We found that (1) ammonium uptake of Landsberg erecta was higher than that of Columbia, when ammonium was supplied at higher concentration, and (2) cytosolic glutamine synthetase 1;2 was highly increased by ammonium supply in the root of Landsberg erecta. The present study suggested the importance of these two factors for adaptation of Arabidopsis to an ammonium-rich environment.

Key words:
ammonium, Arabidopsis, ecotype, glutamine synthetase, growth

Received 19 Feb. 2017;
Accepted 28 Mar. 2017

doi.org/10.1080/00380768.2017.1314178

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Introduction

Nitrogen availability often limits the growth of plants because plants require nitrogen for several important compounds, including protein, amino acids, chlorophyll, and nucleic acids (Marschner 1995). Plants can assimilate both nitrate and ammonium from the environment, which are used for plant growth (von Wirén et al. 2000). Ammonium requires less energy for uptake and assimilation than nitrate, mainly because nitrate has to be reduced to ammonium prior to assimilation (von Wirén et al. 2000). Although the ammonium concentration is lower than the nitrate concentration in most soils (Miller et al. 2007), ammonium can be the dominant nitrogen source in natural and agricultural ecosystems (Britto and Kronzucker 2002). It has been shown that excess ammonium supply to plants and ammonium provided as a sole nitrogen source often inhibits plant growth (Esteban et al. 2016).

The inhibitory effect of ammonium masks its importance (Britto and Kronzucker 2002), despite this nutrient being a good nitrogen source for plant growth. Much research in recent years has focused on ammonium toxicity and ammonium nutrient use. The usage efficiency of nitrogen is determined by two factors: transport and assimilation (Good et al. 2004). Ammonium transport in the plant root comprises two systems: (1) the high-affinity ammonium transport system (HATS) and (2) the low-affinity ammonium transport system (LATS) (Rawat et al. 1999). The HATS carries ammonium below millimolar concentrations, and the HATS molecule is an ammonium transporter (AMT; von Wirén et al. 2000). The LATS transports ammonium in a concentration-dependent manner. Recent work suggested that the LATS molecule is an ammonium facilitator (AMF; Chiasson et al. 2014). For ammonium assimilation, glutamine synthetase (GS, also known as GLN) plays a vital role because it assimilates ammonium into glutamate (Lea and Miflin 1974). The GS family is divided into two groups by intracellular localization (Miflin and Habash 2002): GS1 and GS2. GS1 localizes in the cytoplasm, whereas GS2 localizes in the chloroplast. Arabidopsis expresses four GS1 genes in the root (Ishiyama et al. 2004).

Previous genetic approaches revealed the mechanisms and the components involved in ammonium toxicity (Qin et al. 2008, Barth et al. 2010, Li et al. 2010). These studies discussed the toxicity of ammonium at 20 to 60 mM concentration with a sufficient nitrate supply. Other reverse genetic approaches revealed the importance of ammonium assimilatory enzymes. The functional disruption of ammonium assimilatory enzymes GLN1;2 (Lothier et al. 2011, Funayama et al. 2013, Guan et al. 2016, Konishi et al. 2017) and NADH-dependent glutamate synthase (also known as glutamine oxoglutarate aminotransferase (GOGAT); Tamura et al. 2010, Kojima et al. 2014, Konishi et al. 2014), leads to the decrease of ammonium use in plants. These studies analyzed the responses of plants to ammonium at relatively lower concentrations (e.g. at 1 mM) to understand the mechanism for ammonium use. The purpose of this study is to understand the mechanism for the adaptation in ammonium environment. We compared the growth of two *Arabidopsis thaliana* ecotypes, Columbia (Col) and Landsberg *erecta* (Ler). The ammonium uptake was measured in the root grown in hydroponic culture. We also examined the the expression levels of ammonium assimilatory enzymes in roots using real-time polymerase chain reaction (PCR) analysis. The present study suggested the two differences between Col and Ler: (1) ammonium uptake at high ammonium concentration, (2) the response of cytosolic glutamine synthetase 1;2 to ammonium supply. They are key factors for adaptation of Arabidopsis to an ammonium-rich environment.

Materials and Methods

Plant material and growth analysis

Seeds of the Arabidopsis thaliana ecotypes Bay-0 (CS954), En-T (CS6176), Ms-0 (CS6797), Nd-1 (CS1636), and Van-0 (CS6884) were provided by the Arabidopsis Biological Resource Center (Ohio Univ., OH, USA).

Seeds of Arabidopsis ecotypes were germinated on rock wool. After 4 d, plants were thinned and transferred to a hydroponic solution as described previously for shoot:root analysis (Konishi et al. 2017), and for uptake analysis (Loqué et al. 2006). For shoot:root analysis,

plants were cultured in the hydroponic solution containing 1 mM NH_4Cl and 10 μM KNO_3 buffered with 5 mM 2-(N-morpholino) ethanesulfonic acid (MES) adjusted to a pH of 5.8 with KOH for 6 weeks. For uptake analysis, plants were pre-cultured in the hydroponic nutrient solution containing 2 mM NH_4NO_3 for 6 weeks, following which they were transferred to the nutrient solution containing no nitrogen for 3 days. The hydroponic solution was exchanged twice weekly. Plants were grown in a climate chamber (Biotron LPH-350S, Nippon Medical and Chemical Instruments Co., Ltd., Tokyo, Japan; 10 h/14 h light/dark, 22°C, 60% humidity, and 160 μmol m^{-2} s^{-1} light intensity). Each pot was aerated by pumping. The roots and shoots were harvested separately. Roots were washed in 1 mM $CaSO_4$ solution for 1 min before harvest. Samples were dried in an oven at 80°C for 7 d and weighed with an electronic balance (XS Analytical Balances, Mettler-Toledo International Inc., Columbus, USA). Experiments were repeated at least twice with similar results, and representative values of one experiment are shown.

^{15}N uptake analysis

The uptake of ^{15}N-labeled NH_4^+ was measured after rinsing the roots in 1 mM $CaSO_4$ for 1 min. The roots were transferred to the solution containing either 0.2 or 2 mM ^{15}N-labeled NH_4^+ (95 atom% ^{15}N). The incubation period was 6 min. The roots were rinsed again in 1 mM $CaSO_4$ for 1 min, and harvested and dried at 80°C for 7 d. The root samples were ground by a Tissue Lyser II (QIAGEN K. K., Tokyo, Japan), and 1.5 mg of sample was used for isotope analysis.

Elemental analyzer and isotope ratio mass spectrometry

Nitrogen and carbon were determined with an elemental analyzer (Flash2000, Thermo Fisher Scientific K. K., Yokohama, Japan). The isotope ratio of nitrogen was determined by isotope ratio mass spectrometry (Delta V Advantage, Thermo Fisher Scientific K.K.).

Real-time PCR

Plants were cultured by two methods: hydroponic culture and vertical agar culture. For the hydroponic culture, plants were grown in a hydroponic solution containing 2 mM NH_4NO_3 (Loqué et al. 2006) for 6 weeks. Plants were transferred to the solution containing no nitrogen and grown for 3 d, following which they were transferred to the solution containing either 0.2 or 2 mM NH_4Cl for 24 h. For the vertical agar culture, the plants were grown in a climate chamber controlled at 22°C with 60% relative humidity under a 16 h/8 h light/dark cycle, as previously reported (Ishiyama et al. 2004). After 14 d pre-culture with MGRL medium (Fujiwara et al. 1992) containing 7 mM nitrate as the sole nitrogen source, the plants were transferred to the nitrogen-free medium and grown for 3 d. The plants were transferred again to the medium containing 10 mM ammonium as the sole nitrogen source. The roots were harvested at 0, 3, 6, and 12 h after the transfer. In both culture methods, the roots were harvested and frozen in liquid nitrogen. The root samples were stored at −80°C until RNA extraction. Total RNA was prepared with the RNeasy plant mini kit (QIAGEN K.K.). The same amount of total RNA was reverse transcribed with the PrimeScript® reverse transcription (RT) reagent kit with genomic DNA (gDNA) Eraser (Takara Bio Inc., Otsu, Japan). Quantitative real-time PCR analysis was conducted using gene-specific primers as previously described (Ishiyama et al. 2004, Yuan et al. 2007a, Konishi et al. 2014) with SYBR® Premix Ex Taq™ II (Takara) using the Light Cycler® 480 (Roche Diagnostics K.K., Tokyo, Japan). Constitutive expression of ubiquitin (UBQ2) was used to standardize the signal intensity (Ishiyama et al. 2004).

Statistics

Microsoft Excel, with the add-in software Ekuseru-Toukei (Social Survey Research Information Co., Ltd. Tokyo, Japan) was used for the statistical analysis.

Results

Arabidopsis ecotypes, Col and Ler showed different response to a high ammonium condition

In the present study, we sought to identify a response of Arabidopsis ecotypes, Col and Ler to ammonium nutrition. In addition, T-DNA insertion line for cytosolic glutamine synthetase 1;2 (*gln1;2-1*, Col background) was cultured at the same time as a control, since we have already shown that *gln1;2-1* grew slower than wild-type under 1 mM ammonium condition (Konishi et al. 2017). Plants were grown in

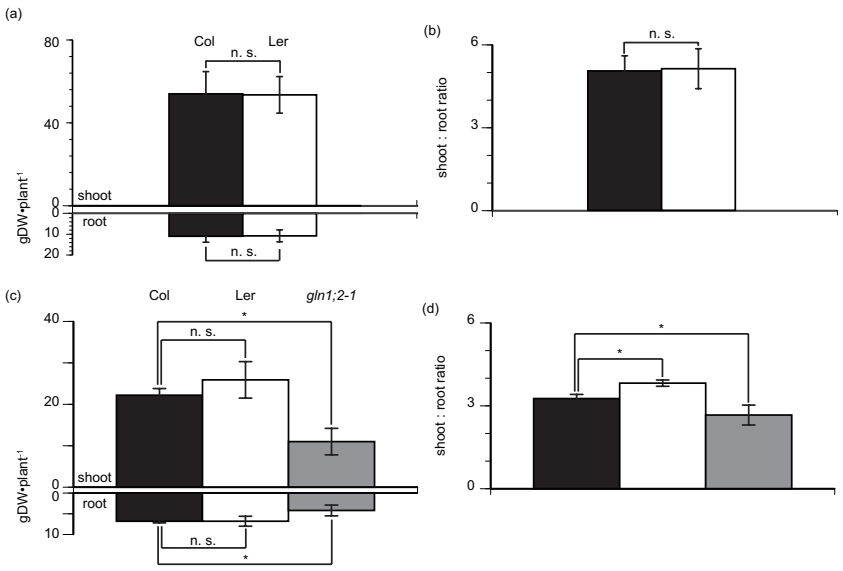

Figure 1 Growth of Col, Ler and T-DNA insertion lines for *GLN1;2* in hydroponic culture solution containing nitrate or ammonium as a major nitrogen source

Dry weight (a) and the ratio of shoot dry weight to root dry weight (b) of the shoot and root of Col (closed column) and Ler (open column) grown in hydroponic solutions containing 0.5 mM KNO_3 as the sole nitrogen source for 3 weeks after the pre-culture in hydroponic solutions containing 2 mM NH_4NO_3 for 6 weeks. Bars indicate means ± standard deviation (SD) (n = 6). Significant differences, identified by the Student's *t*-test, are marked with asterisks: n. s. indicates not significant.

Dry weight (c) and the ratio of shoot dry weight to root dry weight (d) of the shoot and root of Col (closed column), Ler (open column) and T-DNA insertion lines for *GLN1;2* (gray column) grown in hydroponic solutions containing 1 mM NH_4Cl as the major nitrogen source supplemented with 10 μM nitrate for 6 weeks. Bars indicate means ± standard deviation (SD) (n = 6). Significant differences between wild type and mutant, identified by the Student's t-test, are marked with asterisks: $*p < 0.005$, n. s. indicates not significant.

the hydroponic nutrient solution containing 1 mM NH_4Cl and 10 μM KNO_3 and buffered with 5 mM MES adjusted to a pH 5.8 with KOH for 6 weeks (Konishi et al. 2017). Figure 1 illustrates the growth response of Col and Ler at 0.5 mM nitrate or at 1 mM ammonium. Neither biomass nor shoot:root ratio showed significant difference between Col and Ler at nitrate supply (Figs. 1a and 1b). There was no clear difference in biomass between Col and Ler, while the biomass of *gln1;2-1* was significantly smaller than that of wild-type (Fig. 1c). The shoot:root ratio of Ler was significantly higher than that of Col, conversely, the shoot:root ratio of gln1;2-1 was significantly lower than that of Col (Fig. 1d).

Ammonium uptake at higher ammonium concentration of Ler was higher than that of Col

Since ammonium is transported into the cells of roots, we compared the ammonium uptake of 2 mM ammonium in some ecotypes. Ler showed higher uptake capacities for ammonium at higher ammonium concentration. Figure 2 shows the ^{15}N labeled ammonium uptake and the expression of ammonium transporter (AMT) genes in Col and Ler. Plants were grown hydroponic nutrient solution containing 2 mM NH_4NO_3 for 6 weeks and then they were transferred to the nutrient solution contains no nitrogen for 3 days. An uptake study of ^{15}N labeled ammonium was conducted at 2 mM NH_4Cl (Fig. 2a). The uptake of 2 mM ammonium ranged from 270 to 560 μmoles $h^{-1}g^{-1}DW$. We focused on Col and Ler, since these ecotypes showed highest and lowest ammonium uptake at 2 mM. The ammonium uptake at 0.2 mM indicated that there was no significant difference in ^{15}N-labeled ammonium uptake at a concentration of 0.2 mM (Fig. 2b). However, Ler had an approximately two-fold higher ammonium uptake capacity than Col at a concentration of 2 mM (Fig. 2b). To verify the difference between Col and Ler in ammonium uptake, the transcriptional levels of AMT were compared. The gene expression was measured by quantitative PCR (qPCR). *AMT1;1* and *AMT1;3* were the most abundant AMT genes in both ecotypes (Figs. 2c and 2e). Conversely, the expression of *AMT1;5* was lowest among AMT genes expressing in roots (Fig. 2f). None of the AMT genes showed clear difference between

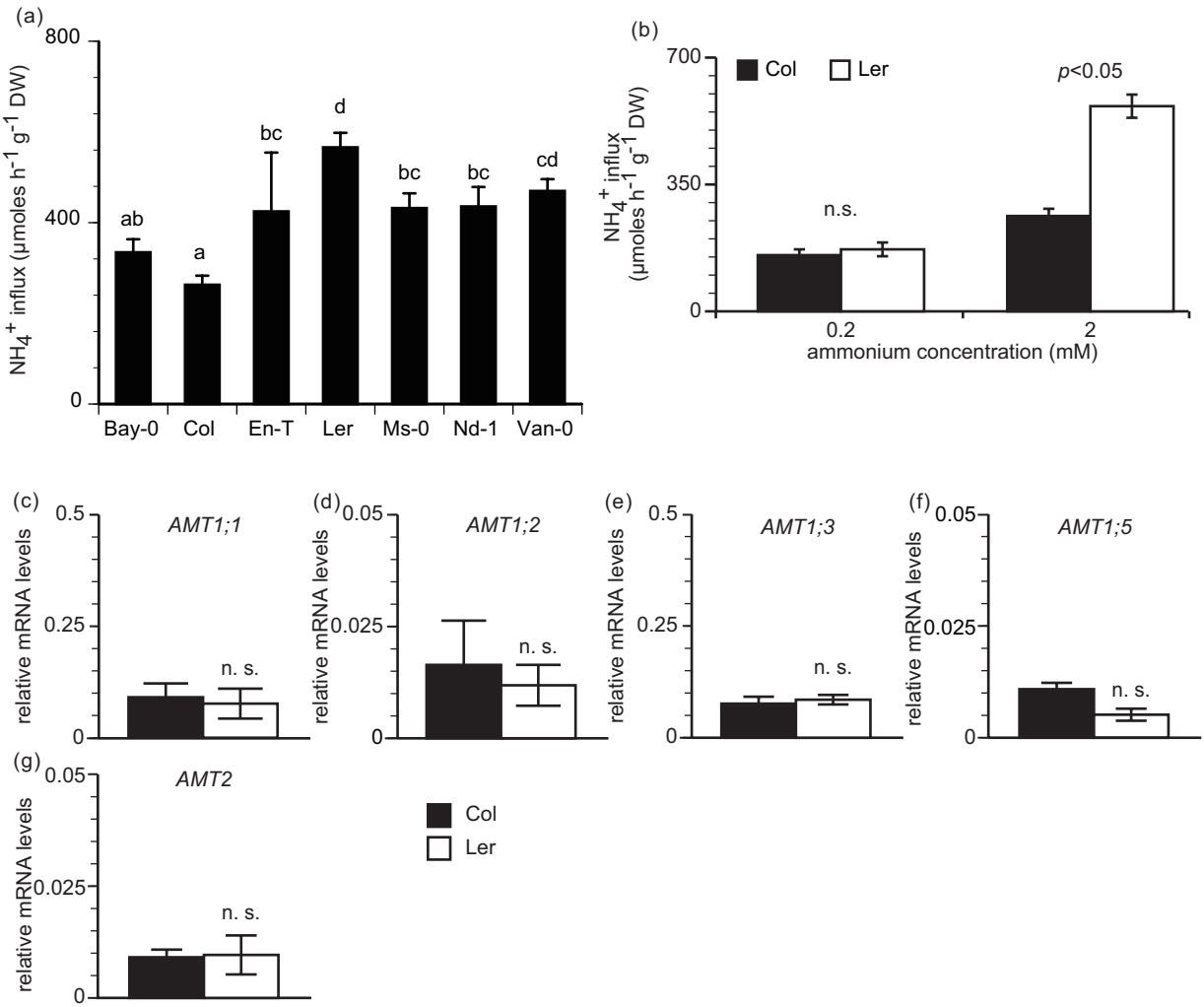

Figure 2 Uptake of ^{15}N-labeled ammonium and the expression of ammonium transporter in roots of 6-week-old Arabidopsis ecotypes

Uptake of ^{15}N labeled ammonium in the Arabidopsis thaliana ecotypes Bay-0, Col, En-T, Ler, Ms-0, Nd-1, and Van-0. ^{15}N-labeled ammonium was supplied at 2 mM. Bars indicate means ± standard deviation (SD). Individual root samples were taken from six plants. One-way analysis of variance (ANOVA) followed by Bonferroni tests were used, and significant differences at $P < 0.05$ within each group are indicated by different letters.

Uptake of ^{15}N labeled ammonium (b) in Col (closed column) and Ler (opened column). ^{15}N-labeled ammonium was supplied at either 0.2 or 2 mM. Bars indicate means ± standard deviation (SD). Individual root samples were taken from six plants.

The transcriptional levels of ammonium transporter (AMT, c–g). *AMT1;1* (c), *AMT1;2* (d), *AMT1;3* (e), *AMT1;5* (f), and *AMT2* (g) were determined in the roots.

Bars indicate means ± SD (n = 5 plants).

Plants were grown in a hydroponic solution containing 2 mM NH_4NO_3 for 6 weeks, following which they were transferred to the solution containing no nitrogen for 3 d.

Col and Led in roots (Fig. 2).

GLN1;2 was rapidly increased by ammonium supply in the root of Ler

To investigate the response to ammonium supply, 0.2 or 2 mM NH_4Cl was supplied to plants in a hydroponic culture. The expression of *GS/GOGAT* was investigated with qPCR in roots 24 h after ammonium supply. Figure 3 shows the response of *GLN* genes to ammonium supply in hydroponic culture. The expression of *GLN1;2* was highest among *GLN* genes in roots (Fig. 3b). At 2 mM ammonium supply, the expression of *GLN1;2* in Ler was double of that in Col (Fig. 3b). Conversely, the expression of *GLN1;3* and *GLN1;4* in Ler was almost half of that in Col at 0.2 mM ammonium supply (Figs.

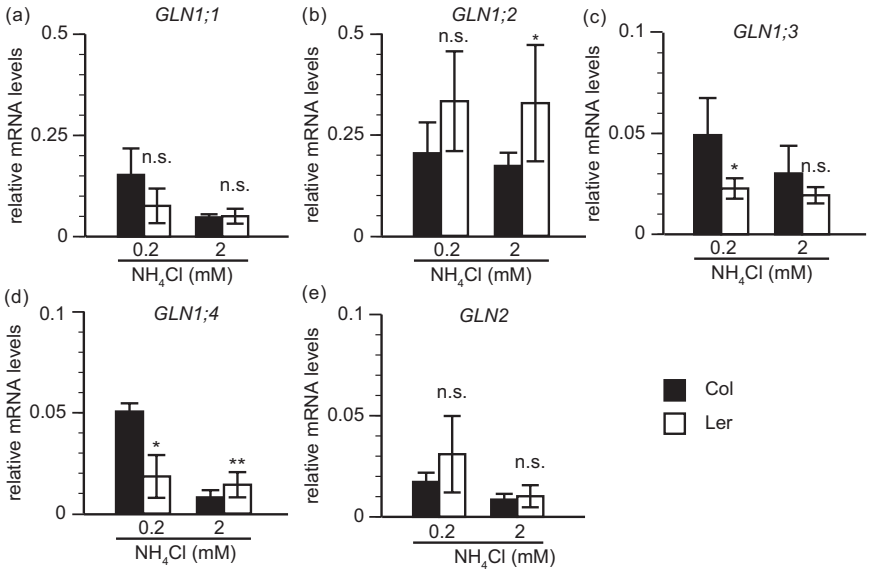

Figure 3 Ammonium re-supply changes the transcriptional levels of glutamine synthetase in roots

Plants were grown in hydroponic solution containing 2 mM NH_4NO_3 for 6 weeks, following which they were transferred to the solution containing no nitrogen for 3 d, and then to the solution containing either 0.2 mM or 2 mM NH_4Cl for 24 h. The transcriptional levels of *GLN1;1* (a), *GLN1;2* (b), *GLN1;3* (c), *GLN1;4* (d), and *GLN2* (e) were determined in the roots. Bars indicate means ± SD (n = 5 plants).

3c and 3d). *GLN1;1* and *GLN2* were not strongly influenced by ammonium supply in both ecotypes (Figs. 3a and 3e). Figure 4 shows the response of *GOGAT* genes to ammonium supply in hydroponic culture. Neither *Fd-GOGAT2* nor *NADH-GOGAT* show a clear response to ammonium supply (Fig. 4), and there was no difference in the expression of *GOGAT* genes between Col and Ler (Fig. 4). The expression of *AMT1;5* in Ler was higher than that in Col at 2 mM ammonium supply (Fig. 5d), while that of *AMT1;2* in Ler was lower than that in Col (Fig. 5b). Neither *AMT1;1* nor *AMT1;3* showed significant difference in the expression between Col and Ler at 2 mM ammonium (Figs. 5a and 5c). To investigate the time-dependent response to ammonium supply, the expression of *GLN1;2* was measured in the plants grown on vertical agar culture after 10 mM NH_4Cl supply. Figure 6 shows the time-dependent accumulation of *GLN1;2*. The expression of *GLN1;2* in Ler responded to ammonium supply more rapidly, resulting it was always higher than that in Col after ammonium supply (Fig. 6).

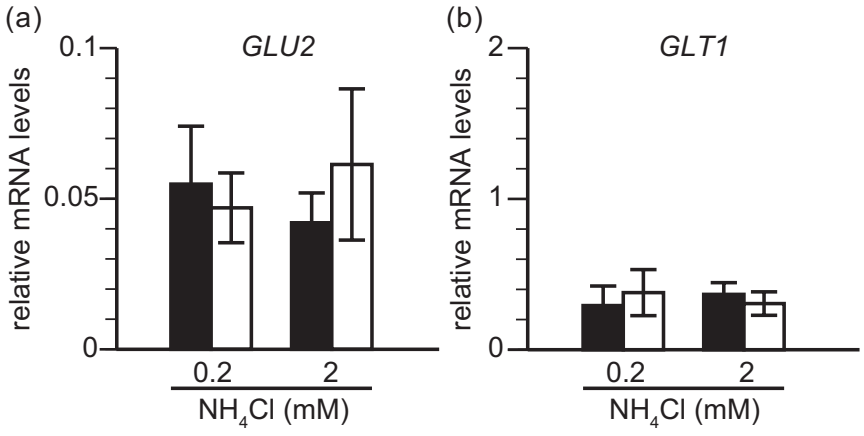

Figure 4 Ammonium re-supply changes the transcriptional levels of glutamate synthase in roots

Plants were grown in hydroponic solution containing 2 mM NH_4NO_3 for 6 weeks, following which they were transferred to the nitrogen-free solution for 3 d, and finally transferred to the solution containing either 0.2 mM or 2 mM NH_4Cl for 24 h. The transcriptional levels of *GLU2* (*Fd-GOGAT2*) (a), and *GLT* (*NADH-GOGAT*) (b) are shown. Bars indicate means ± SD (n = 5 plants).

Discussion

The growth comparison of Arabidopsis ecotypes under various nitrogen regimes allowed for the identification of natural variation of Arabidopsis in nitrogen use (Ikram et al. 2012, Sarasketa et al. 2014). It has been shown that there is a clear negative correlation between shoot ammonium content and shoot biomass, whereas there is no correlation between shoot biomass and nitrogen assimilatory enzymatic activities (Sarasketa et al. 2014). Recent reverse genetic studies indicated that a loss of function in root GS/GOGAT isozymes markedly

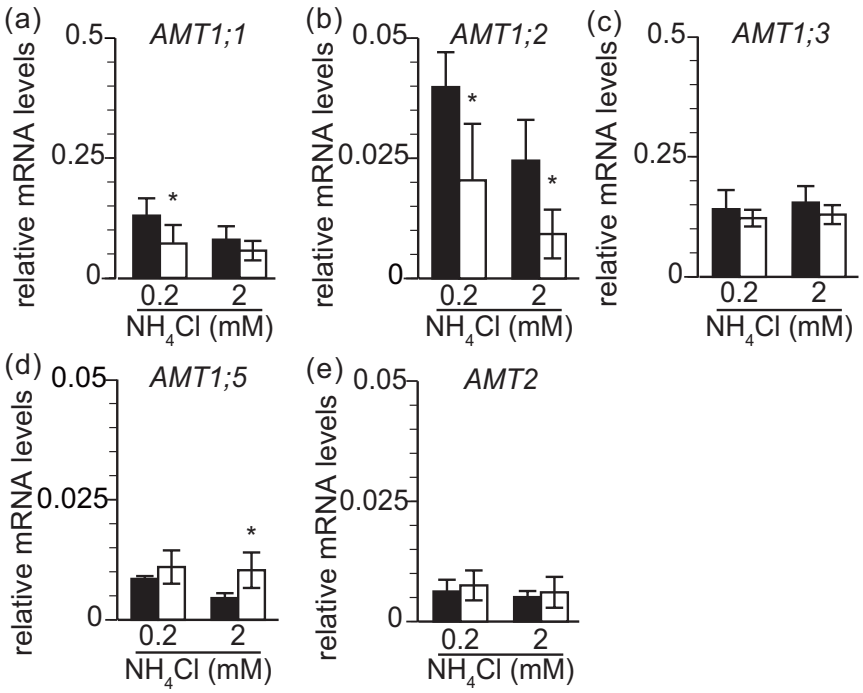

Figure 5 Ammonium re-supply changes the transcriptional levels of ammonium transporter in roots

Plants were grown in hydroponic solution containing 2 mM NH_4NO_3 for 6 weeks, following which they were transferred to the nitrogen-free solution for 3 d, and finally transferred to the solution containing either 0.2 mM or 2 mM NH_4Cl for 24 h. The transcriptional levels of *AMT1;1* (a), *AMT1;2* (b), *AMT1;3* (c), *AMT1;5* (d), and *AMT2* (e) are shown. Bars indicate means ± SD (n = 5 plants).

reduced the growth of the plant, suggesting the importance of the root in ammonium assimilation (Tamura et al. 2010, Funayama et al. 2013, Konishi et al. 2014). Although ammonium translocation to the shoot was increased by exposure to high levels of ammonium (Schjoerring et al. 2002), this could not exclude the importance of the root. Therefore, it is useful to determine whether the function of the root can be related to the natural variation in ammonium use in Arabidopsis (Sarasketa et al. 2014). The present study set out to investigate the contribution of the root in adaptation of Arabidopsis for an ammonium-rich environment and shows that ammonium uptake and the expression of the ammonium assimilatory gene are tightly related to the adaptation to an ammonium-rich environment. In this work, Col and Ler were grown in the hydroponic solution containing 1 mM ammonium as a major nitrogen source. In previous work, we have developed a hydroponic culture condition which allows to evaluate the growth of Arabidopsis in ammonium nutrition (Konishi et al. 2017). T-DNA insertion line for *GLN1;2* was used as a control (Fig. 1), it showed a marked decrease in dry weight, compared with its genetic background Col. The shoot:root ratio of *gln1;2* was lower than that of Col (Fig. 1d). A decrease in the shoot:root ratio in response to a reduced availability of nitrogen is typical, for all plant species and cultural methods (Ericsson 1995). Therefore, the decrease of shoot:root ratio confirms the reduced availability of ammonium in *GLN1;2* T-DNA insertion line. Ammonium supply induces the expression of *GLN1;2* in roots (Ishiyama et al. 2004), and the T-DNA insertion in *GLN1;2* leads a plant to growth retardation under ammonium condition, thus GLN1;2 is essential for ammonium assimilation in roots (Lothier et al. 2011, Konishi et al. 2017). Although the dry weight of Ler did not show clear difference from Col, the shoot:root ratio of Ler was significantly higher than that of Col (Fig. 1d). Conversely, the shoot:root ratio of Ler was higher than that of Col, suggesting an increased availability of ammonium in Ler. Notably, there was no significant difference in shoot:root ratio under nitrate condition (Fig. 1b). The isotope-labeled ammonium uptake study indicated that the ammonium uptake in Ler was higher than that in Col at 2 mM ammonium supply (Fig. 2a). The qPCR indicated that there were no significant differences between Col and Ler in the expression of AMT genes in roots (Figs. 2 b–2f). Consistently, there was no significant difference between Col and Ler in ammonium uptake at 0.2 mM ammonium supply (Fig. 2a). AMT has been known to be regulated at both post-transcriptional (Yuan et al. 2007b) and post-translational (Lanquar et al. 2009) levels. Therefore, higher ammonium uptake capacity in Ler at higher ammonium concentration might account for the varieties of the factors those related to

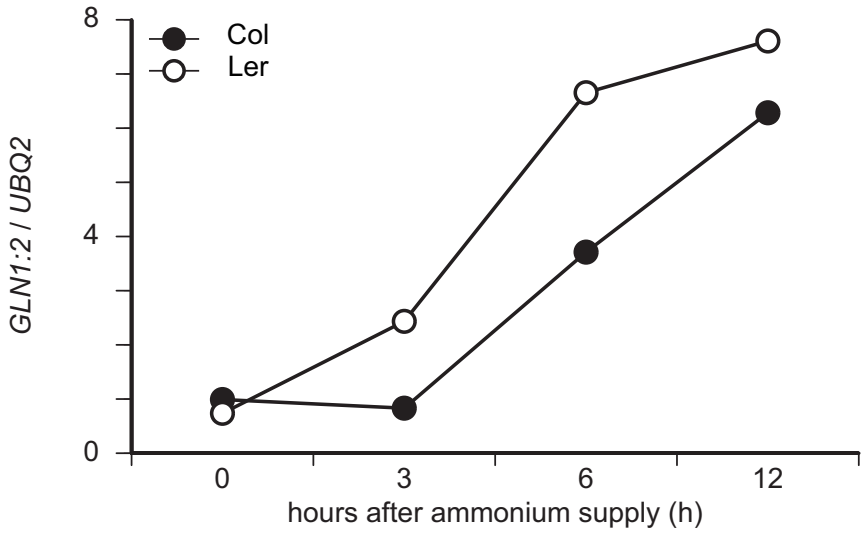

Figure 6 Time dependent change of *GLN1;2* mRNA in roots of Col and Ler on vertical agar plate

After the 14 d in pre-culture with MGRL medium (Fujiwara et al. 1992) containing 7 mM nitrate as a sole nitrogen source, the plants were transferred to the nitrogen-free medium and grown for 3 d. The plants were transferred again to the medium containing 10 mM ammonium as a sole nitrogen source. The roots were harvested at 0, 3, 6, and 12 h after the transfer. Paired t-test indicated the significant difference between Col and Ler.

the AMT regulation, or for the ammonium transport mechanism not yet determined. The capacity for ammonium accumulation in the shoot is one of the key adaptation factors for Arabidopsis ecotypes in a higher ammonium environment (Sarasketa et al. 2014). Our results provide the first evidence for Arabidopsis ecotypes having different ammonium uptake capacity. Future work should focus on the discovery of the transport mechanism responsible for higher ammonium concentration and/or the regulatory mechanism.

When ammonium was supplied to the plant, the induction of *GLN1;2* in Ler was higher (Figs. 3 and 4) and faster (Fig. 4) than that in Col. Since *GLN1;2* is an essential gene for ammonium assimilation (Lothier et al. 2011, Funayama et al. 2013, Konishi et al. 2017), the response of root *GLN1;2* may account for the higher shoot:root ratio of Ler in an ammonium environment. The comparison of *GLN1;2* genome (Alonso-Blanco et al. 2016) allowed to find out two single nucleotide polymorphisms (SNPs). The impact of both SNPs was modifier, and they are intron variant, a transcript variant occurring within an intron. There was no other SNPs found in coding region or promoter region. Intron variant could participate in the transcriptional regulation (Greenwood and Kelsoe 2003). Future work may focus on the effects of these intron variants found in this study on ammonium dependent *GLN1;2* expression. The presented dataset suggests GLN1;2-dependent adaptation to a high ammonium condition by Arabidopsis ecotypes. However, the relationship between GLN1;2 and ammonium transport, particularly the relationship between GLN1;2 and higher ammonium uptake capacity, remains to be determined. There are several lines of evidences supporting the relationship between GS and ammonium transport. First, the nitrogen supply-dependent decrease of AMT1 disappears in the presence of methionine sulfoximine (MSX), an inhibitor of GS (Rawat et al. 1999). Second, soybean nodule 26, a member of the major intrinsic protein/aquaporin superfamily, is able to associate with GS, suggesting a promotion of efficient assimilation of fixed nitrogen and prevention of ammonium toxicity (Masalkar et al. 2010). An additional direction for future studies is to determine the regulatory effects of GS1 on ammonium transport.

Acknowledgments

We are grateful to Mrs. Ikumi Sakurada-Enomoto for technical assistance and figure preparation. Japan Advanced Plant Research Network supported by JSPS is also acknowledged for the use of Elemental Analyzer.

Disclosure statement

Conflicts of interest: No conflicts of interest declared

Funding

JSPS KAKENHI Grant Numbers, 21688006 and 26450073 to SK supported this study.

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