Contributions of two cytosolic glutamine synthetase isozymes to ammonium assimilation in Arabidopsis roots

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Contributions of two cytosolic glutamine synthetase isozymes to ammonium assimilation in *Arabidopsis* roots

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Glutamine synthetase (GS) catalyzes a reaction incorporating ammonium into glutamate and yielding glutamine in cytosol and chloroplasts. Although the enzymatic characteristics of GS1 isozymes are well known, their physiological functions in ammonium assimilation and regulation in roots remain unclear. We show here evidence that two cytosolic GS1 isozymes (GLN1;2 and GLN1;3) contribute to ammonium assimilation in *Arabidopsis* roots. *Arabidopsis* T-DNA insertion lines for *GLN1;2* and *GLN1;3* (i.e. *gln1;2* and *gln1;3* single mutants), the *gln1;2*:*gln1;3* double mutant, and the wild-type accession (Col-0) were grown in hydroponic culture with variable concentrations of ammonium to compare their growth, and their content of nitrogen, carbon, ammonium, and amino acids. *GLN1;2* and *GLN1;3* promoter-dependent green fluorescent protein was tested under ammonium-supplied or -nonsupplied conditions. Loss of *GLN1;2* caused significant suppression of plant growth and glutamine biosynthesis under ammonium-replete conditions. Conversely, loss of *GLN1;3* caused slight defects in growth and Gln biosynthesis that were only visible based on comparison of *gln1;2* single and *gln1;2:gln1;3* double mutants. *GLN1;2* as being the most abundantly expressed GS1 isozyme markedly increased following ammonium supply and its promoter activity was localized at the cortex and epidermis, while *GLN1;3* was lowly expressed at the pericycle, respectively, suggesting their different physiological contributions to ammonium assimilation in roots. The *GLN1;2* promoter-deletion analysis identified regulatory sequences required for controlling ammonium-responsive gene expression of *GLN1;2* in *Arabidopsis* roots. These results shed light on *GLN1* isozyme-specific regulatory mechanisms of *Arabidopsis* adaptation to the ammonium-replete environment.

Introduction

Ammonium and nitrate are inorganic nitrogen forms used in plant growth (von Wirén *et al*., 2000). Plants may preferentially take up ammonium for energy conservation when both nitrate and ammonium are present (Gazzarrini *et al*., 1999; Gu *et al*., 2013; Sasakawa and Yamamoto, 1978). However, given that excessive ammonium supply may inhibit plant growth (Britto and Kronzucker, 2002; Hachiya *et al*., 2012; von Wirén *et al*., 2000), ammonium must be quickly assimilated into glutamine (Andrews *et al*., 2013; Yamaya and Kusano, 2014). The glutamine synthetase/glutamate synthase (GS/GOGAT) cycle is the key step in ammonium assimilation in higher plants (Lea and Azevedo, 2007; Tobin and Yamaya, 2001). Glutamine synthetase (GS or GLN) catalyzes a reaction incorporating ammonium into glutamate and generating glutamine as a product in an adenosine triphosphate (ATP)-dependent manner (Tobin and Yamaya, 2001). Glutamate synthase (also termed glutamate 2-oxoglutarate aminotransferase, GOGAT) transfers the amine group in the amide side chain of glutamine to 2-oxoglutarate, yielding two molecules of glutamate; one molecule serves as a substrate for GS, whereas the other is used for transport, storage, or further metabolism (Tobin and Yamaya, 2001). GS is categorized into

Abbreviations:

GFP, green fluorescent protein; GOGAT, glutamate synthase; GS, glutamine synthetase; qPCR, quantitative real-time PCR; RT, reverse transcription; T-DNA, transfer DNA; UBQ, ubiquitin; UI, usage index; UpE, uptake efficiency; UPLC, ultra performance liquid chromatography

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two groups: 1) the cytosol-localized GS1 group and; 2) the GS2 group localized mainly in chloroplasts (Swarbreck *et al*., 2011). In the *Arabidopsis* genome, a single *GLN2* gene and five *GLN1* genes are encoded. A barley mutant lacking functional GS2 does not grow normally under ambient-CO_2 conditions; however, this growth defect is rescued under high CO_2 conditions (Blackwell *et al*., 1988). Thus, it has been suggested that GS2 could assimilate the ammonium derived from photorespiration (Wallsgrove *et al*., 1987), whereas GS1 isozymes assimilate non-photorespiratory ammonium (Tobin and Yamaya, 2001). Besides primary uptake and photorespiration, ammonium can originate in several metabolic processes, including nitrate reduction, phenylpropanoid metabolism, degradation of transported amides, and protein catabolism (Li *et al*., 2014; Schjoerring *et al*., 2002). Four GS1 isozymes encoded by *GLN1;1*, *GLN1;2*, *GLN1;3* and *GLN1;4* of Arabidopsis that have been identified to have different enzymatic characteristics when they are expressed in *E. coli* (Ishiyama *et al*., 2004). Individual GS1 isoenzymes may share assimilatory functions for the ammonium originating in non-photorespiration (Yamaya and Kusano, 2014). Analysis of mutants lacking a specific GS1 isozyme suggests that GS1 functions in non-photorespiratory ammonium assimilation in monocotyledonous crop plants, such as rice (Funayama *et al*., 2013; Tabuchi *et al*., 2005) and maize (Cañas *et al*., 2010; Martin *et al*., 2006). Phylogenetic analysis further suggests key differences between crop and *Arabidopsis* GS1 amino acid sequences (Thomsen *et al*., 2014), while the isogene-specific physiological functions of GS1 in *Arabidopsis* have been only partially documented or studied to be focused on their roles in nitrogen remobilization in aerial organs based on their predominant expression found in vascular tissues (Guan *et al*., 2015; Thomsen *et al*., 2014).

Three previous literatures report on the physiological functions of GS1 isozymes in *Arabidopsis* using reverse-genetic approaches (Guan *et al*., 2015; Guan *et al*., 2016; Lothier *et al*., 2011). *GLN1;2* is essential for nitrogen assimilation and ammonium detoxification (Lothier *et al*., 2011; Guan *et al*., 2016). *GLN1;2* promoter activity is localized mainly in the minor veins of leaves and flowers and GLN1;2 protein is localized in companion cells (Lothier *et al*., 2011). Transfer DNA (T-DNA) insertion lines for *GLN1;2* showed a decrease in GS activity and rosette biomass compared with the wild-type (WT) under nitrate-sufficient conditions; however, but no significant difference in nitrogen remobilization was found. When ammonium was supplied as the sole nitrogen source after the pre-culture in a nitrate-sufficient condition, *GLN1;2* insertion lines developed root hairs and reduced rosette sizes (Lothier *et al*., 2011). Guan *et al*. (2015) reported that *GLN1;2* plays an important role in nitrogen remobilization. Both the single T-DNA insertion line for *GLN1;2* and the double insertion line for *GLN1;1* and *GLN1;2* showed decrease in seed yield, whereas the single insertion line for *GLN1;1* showed yield comparable to the wild type. The *GLN1;2* promoter-dependent green fluorescent protein (GFP) showed fluorescence of GFP localized in the vascular cells of roots, petals, and stamens (Guan *et al*., 2015). A more recent article showed that GLN1;2 is the main isozyme contributing to shoot GS1 activity in the vegetative growth stage and that it can be up-regulated to relieve ammonium toxicity (Guan *et al*., 2016). There remains a need for an efficient method that minimizes the nitrate use in the nutrient solution.

The enzymatic characteristics of recombinant GLN1;2 and GLN1;3 suggest that these two GS1 isozymes with low substrate affinities may contribute to ammonium assimilation in *Arabidopsis* under ammonium-replete conditions (Ishiyama *et al*., 2004). However, the role-sharing of GLN1;2 and GLN1;3 in ammonium-supplied roots has remained to be elucidated. The present study provides evidence that *GLN1;2* and *GLN1;3* are necessary for ammonium assimilation in *Arabidopsis* roots, particularly in roots exposed to high concentrations of ammonium supply, based on results obtained through reverse genetic approached using the T-DNA insertion mutants and the promoter-GFP lines reporting their differential physiological functions and spatiotemporal regulations. The finding of ammonium-responsive regulatory sequences in the *GLN1;2* gene promoter region further implicates a distinct contribution of the GLN1;2 isozyme to ammonium assimilation in roots under ammonium-replete conditions.

Materials and methods

Hydroponic culture

Three to five *Arabidopsis* seeds were germinated on water-moistened rock wool for 4 days in the dark, and single seedlings were selected. Plants were transferred to a hydroponic nutrient solution described by Loqué *et al*. (2006) with modifications. The modified hydroponic solution was buffered with 5 mM 2-(N-morpholino) ethanesulfonic acid (MES) adjusted to a pH of 5.8 with KOH, and 2 mM NH_4NO_3 was removed to be replaced with 10 µM KNO_3 and various concentrations of NH_4Cl, given that a small amount of nitrate alleviates the detrimental effects of pure ammonium nutrition (Garnica *et al*., 2010; Krouk *et al*., 2006). The nutrient solution was always buffered with MES. Plants were grown in three sizes in pre-culture because of space limitations. First, at ammonium concentrations of 0.1, 0.3, 1, 2, 3, 5, and 10 mM, 18 WT plants were grown in a 0.8 L plastic container filled with 0.7 L of nutrient solution (Fig. 1). Second, at ammonium concentrations of 0.1, 0.3, 0.5, and 1 mM, 220 plants (44 plants per line, five compared genotypes) were grown in a 2 L plastic container filled with 2 L of nutrient solution (Fig. 3–5). Third, at ammonium concentrations of 0.1 or 3 mM, 120 plants (17 plants per line, seven compared genotypes) were grown in a 5.9 L plastic container filled with 5 L of nutrient solution (Fig. 7– 9). All plastic containers were purchased from Sanko Co., Ltd, (Tokyo, Japan).

Six to eight plants from the pre-culture were then transferred at 21 to 25 days after sowing to a black acrylic resin plate (0.11 × 0.15 m, 5 mm thick) with nine holes. A 0.8 L plastic container was filled with 0.7 L hydroponic solution and covered with the resin plate. 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 plastic container was aerated by pumping. Roots and shoots were harvested separately 6 weeks after sowing. Roots were washed in 1 mM $CaSO_4$ solution for 1 min before harvest. Samples were collected in an envelope or 2 mL safe-lock tubes (Eppendorf Co., Ltd., Tokyo, Japan) with a zirconia bead. The hydroponic solution was renewed 3 days before harvesting. The harvesting began at 01:00 PM. Samples were frozen in liquid nitrogen immediately after measurement of the fresh weight using CPA324S electronic balance (Sartorius Japan K.K., Tokyo, Japan). Samples for quantitative real-time polymerase chain reaction (qPCR) and amino acid measurements were maintained at −80 °C. Samples for dry weight measurement were dried in an oven at 80 °C for 4–7 days and weighed with an electronic balance (XS Analytical Balances, Mettler-Toledo International Inc, Columbus, USA). Experiments were repeated at least twice obtaining similar results, and representative values of one experiment were shown in Figures.

Cellular localization of GLN1;2 and GLN1;3 promoter activities

The *GLN1;2* upstream region was amplified from Columbia genomic DNA by PCR. KOD plus DNA polymerase (Toyobo Co., Ltd., Osaka, Japan) was used in the PCR with gene-specific primers, *GLN1;2P5697L_F*: (5'-GGGATCCGATG TAGATGATTAAAGATATATAACTA-3') and *GLN1;2P2501L_F*: (5'-CGGATCC

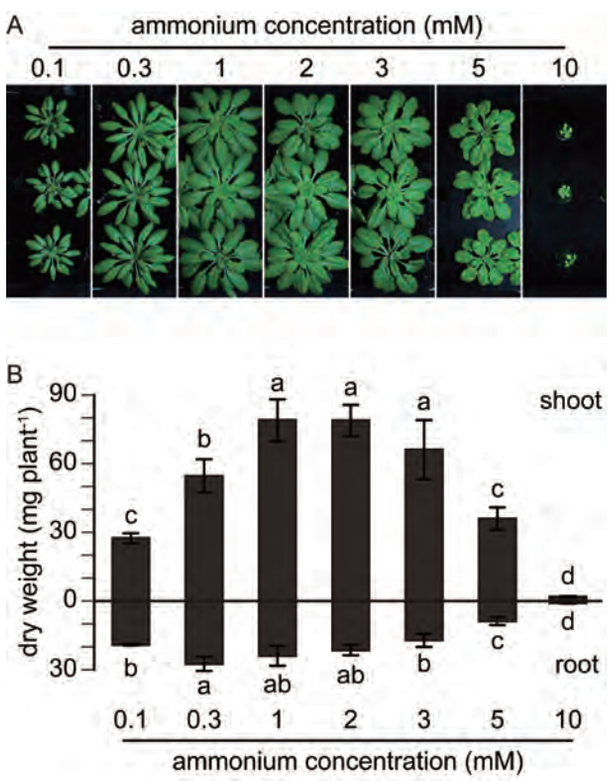


Figure 1.
Growth of wild-type (WT) under increasing concentrations of ammonium (A) Growth of the WT in hydroponic solutions containing 0.1, 0.3, 1, 2, 3, 5, or 10 mM NH_4Cl as the major nitrogen source, supplemented with 10 µM nitrate for 6 weeks. (B) Shoot and root dry weight of the same plants as in A. Bars indicate means ± standard deviation (SD) (n = 4). One-way ANOVA followed by Bonferroni tests were used, and significant differences at p < 0.05 within each group are indicated by different letters.

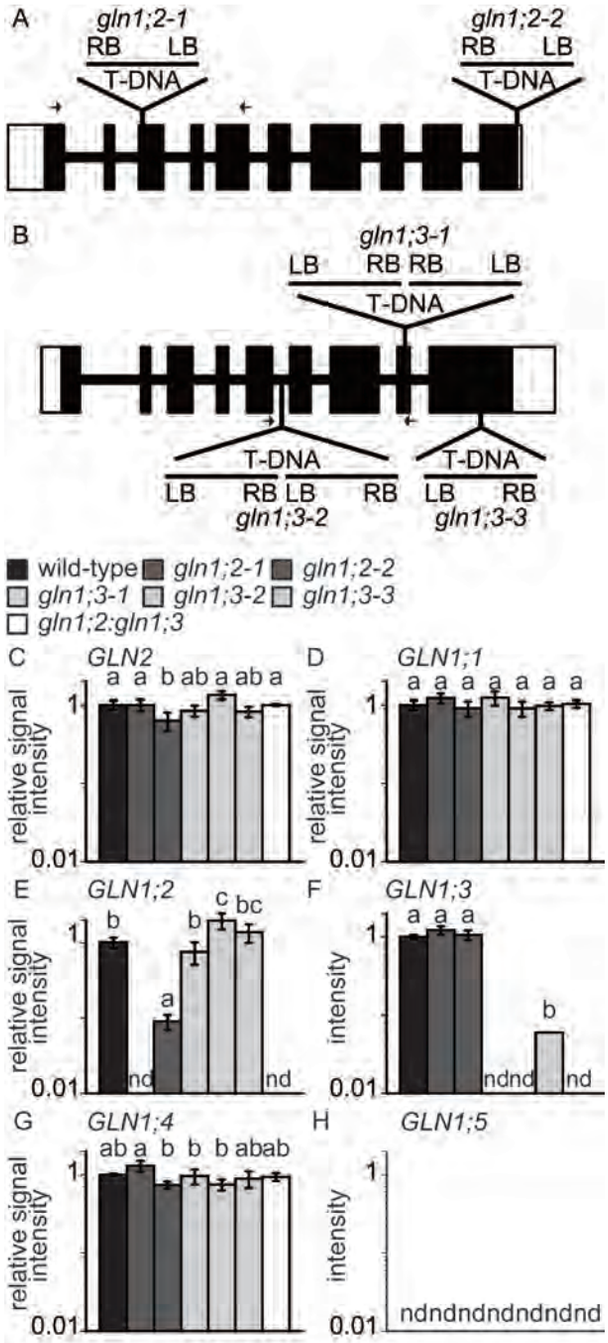


Figure 2.
Isolation of T-DNA insertion lines for *GLN1;2* and *GLN1;3*
(A) The positions of T-DNA insertions in *gln1;2-1* and *gln1;2-2*. (B) The positions of the T-DNA insertion in *gln1;3-1*, *gln1;3-2* and *gln1;3-3*. Exons are indicated as filled boxed regions, whereas lines represent introns and open boxes correspond to *5'*- and *3'*-untranslated sequences. Arrows indicate positions of gene-specific primers used for quantitative polymerase chain reaction (qPCR). (C, D, E, F and G) Quantitative real-time PCR analysis of root RNA from WT (filled columns) and *gln1;2-1*, *gln1;2-2* (dark gray columns), *gln1;3-1*, *gln1;3-2* and *gln1;3-3* (light gray columns), *gln1;2:gln1;3* (opened column) using gene-specific primer sets for *GLN2* (C), *GLN1;1* (D), *GLN1;2* (E), *GLN1;3* (F), *GLN1;4* (G), and *GLN1;5* (H). Plants were grown in hydroponic culture with 1 mM ammonium and 10 μM nitrate for 6 weeks. *UBQ2* was used to standardize the signal intensity. Bars indicate means ± SD (n = 3). One-way ANOVA followed by Bonferroni tests were used, and significant differences at $p < 0.05$ within each group are indicated by different letters.
Fold change in gene expression relative to that of the WT at 1 mM ammonium was determined on the basis of crossing points (CP) values (Pfaffl 2001).

ATTTTAGCAAGAGACCATCCACAC TAAC-3'), paired with a reverse primer, *GLN1;2P_R*: (5'-GCCATGGGGTTGCA AGAAGAAACAAGAAGATTGAA-3'). The region upstream of the *GLN1;2* start codon was tagged with restriction cites for *Bam*HI (GGATCC) and *Nco*I (CCATGG). The entire *GLN1;2* promoter region in different length (5,697 bp or 2,501 bp) was then fused with enhanced GFP (Takara Bio Inc.) using the *Nco*I restriction site designed in the *GLN1;2P_R* primer. The region upstream of the 2,501 bp *GLN1;2* promoter was amplified from genomic DNA by PCR with gene-specific primers, *GLN1;2P5372L_F*: (5'-GAAGCTTCATT TAAGTTTTGTACGACATCTAATT-3'), *GLN1;2P3822L_F*: (5'-GAAGCTTGCG ACAGAAAAAAAGAAAACAAGAC AT-3'), *GLN1;2P3624L_F*: (5'-GAAGC TTTTTTTTTTTTTAGTTTGTCTTTTT TTTT-3'), *GLN1;2P3604L_F*: (5'-GAAG CTTGTCTTTTTTTTTTACCGTCAAC TCTTAC-3'), *GLN1;2P3563L_F*: (5'-GA AGCTTTTCTTAACTGTATGACACC ATTGCTTAC-3'), *GLN1;2P3522L_F*: (5'-GAAGCTTCTGGTAAATTATATTA CCATTTCTATAA-3'), *GLN1;2P3430L_ F*: (5'-GAAGCTTGGCATCTACACTT CATAAAGTGTCGACATC-3'), paired with a reverse primer, *GLN1;2P_R_02*: (5'-GGGATCCTAGACTGCGTGAGAA TGTAAAAATGTAA-3'). The region was tagged with restriction cites for *Hind*III (AAGCTT) and *Bam*HI. The partial fragment of *GLN1;2* promoter region in different length was then fused with the upper region of the 2,501 bp GLN1;2 promoter using *Bam*HI restriction site designed in the *GLN1;2P_R_02* primer. The entire *GLN1;2* promoter region in different length (5,372 bp, 3,822 bp, 3,624 bp, 3,604 bp, 3,563 bp, 3,522 bp or 3,430 bp) was then fused with enhanced GFP (Takara Bio Inc.). The *GLN1;2* promoter-GFP fragment was ligated to pBI101 (Clontech, Palo Alto, CA) based binary vector, as previously reported (Ishiyama *et al*., 2004). The binary plasmids were transferred to *Agrobacterium tumefaciens* GV3101, and *Arabidopsis* plants were transformed according to the floral

dip protocol (Clough and Bent, 1998). *GLN1;3* promoter-GFP lines originate from our previous study (Ishiyama *et al*., 2004).

Plants were grown in hydroponic culture or on vertical agar plates. In the hydroponic culture, plants were grown for 6 weeks in nutrient solution containing 0.1, and 3 mM ammonium and 10 µM nitrate as nitrogen sources. Laser-scanning confocal microscopy was performed with a Nikon C1si System. A CFI Plan Fluor 20× (numerical aperture 0.5; Nikon) or a CFI Plan Apo Lambda 40× (numerical aperture 0.95; Nikon) was used as objective lenses. GFP was excited with the 488 nm line of a multi-argon ion laser. The fluorescence spectra between 500 and 530 nm were obtained with a spectral detector of the Nikon C1si System. Plants were cultured on vertical agar plate in a growth cabinet controlled at 22 °C with 60% relative humidity under 16 h/8 h light/dark cycles, as previously reported (Ishiyama *et al*., 2004). The light intensity used was 40 µmol m^{-2} s^{-1}. Three steps controlled the plant nitrogen nutrition: 1) plants were grown on MGRL agar medium (Fujiwara *et al*., 1992) containing 7 mM nitrate as a major nitrogen source for 14 days; 2) plants were transferred to the nitrogen free MGRL medium and pre-cultured for 3 days to facilitate nitrogen starvation, and 3) plants were then re-transferred to the N-free MGRL medium either supplemented with 10 mM ammonium as the sole nitrogen source or without addition of nitrogen source and incubated for 24 h for confocal microscopy and 9 h for qPCR analysis of GFP expression. Plants were all cultured under sterile conditions. Confocal laser scanning microscopic analysis was performed using a BX61 microscope equipped with a FV500 with a 505–525 nm band pass filter (Olympus, Tokyo, Japan) for detection, as descried previously (Ishiyama *et al*., 2004). Images were processed in Adobe Photoshop.

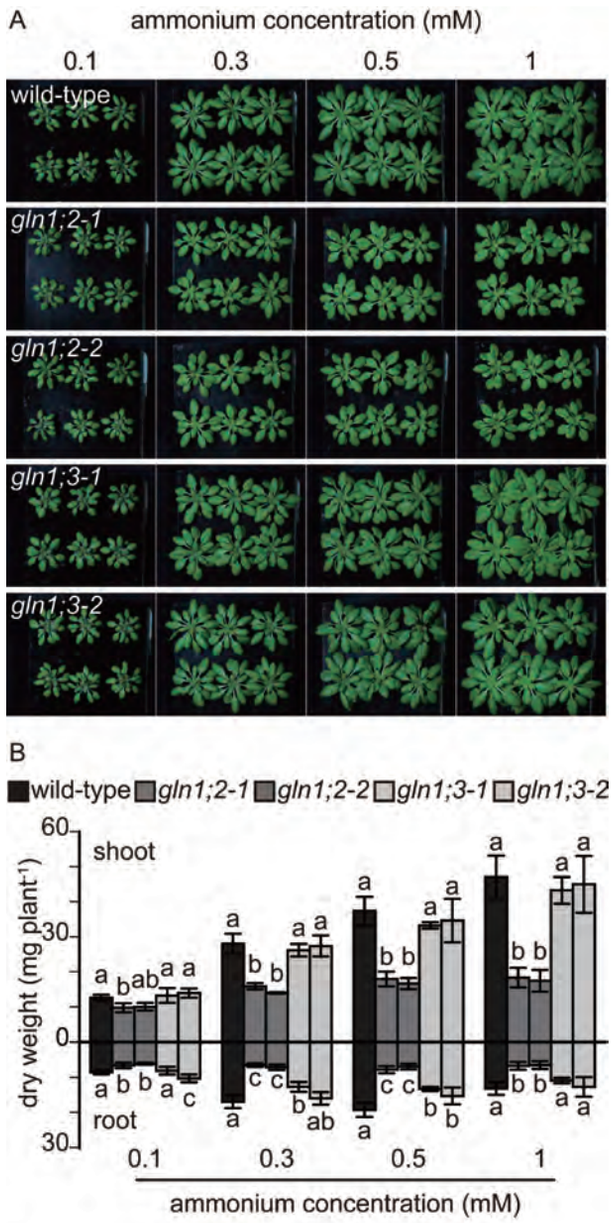


Figure 3.
Growth of the wild-type (WT) and T-DNA insertion lines for *GLN1;2* and *GLN1;3* under low nitrate supply, and the effect of varied ammonium supply in nutrient solution
(A) Phenotype of the WT and insertion lines for *GLN1;2* and *GLN1;3*. (B) Shoot and root dry weights of the WT (filled columns), *GLN1;2* insertion lines (dark gray columns), and *GLN1;3* insertion lines (light gray columns). Plants were grown for 6 weeks in nutrient solutions containing 0.1, 0.3, 0.5, and 1 mM ammonium, and 10 µM nitrate as the nitrogen source. Bars indicate means ± standard deviation (SD) (n = 6). 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.

Quantitative real-time polymerase chain reaction (PCR) analysis and reverse transcription (RT)-PCR analysis

Messenger RNA (mRNA) was quantified by quantitative PCR (qPCR) as previously described (Konishi *et al*., 2014). Plants were hydroponically grown in nutrient solution with 0.1, 1 or 3 mM NH_4Cl and 10 μM KNO_3 for 6 weeks. Total RNA was extracted with an RNeasy Plant Mini Kit (Qiagen, K. K., Tokyo, Japan). Absorbances at 260 nm and 280 nm were measured with a NanoDrop 1000 spectrophotometer (NanoDrop, LMS Co., Ltd. Tokyo, Japan) to quantify and characterize the extracted RNA. RT and DNase treatment were performed using a PrimeScript® RT reagent Kit with genomic (g) DNA Eraser (Takara Bio Inc., Otsu, Japan) with 500 ng of total RNA in a 20 μL final volume, according to the manufacturer's instructions. The products were diluted five times with RNase-free water and used as a template. PCR reactions were performed on a Light Cycler® 480 (Roche Diagnostics K.K., Tokyo, Japan), according to the following program: 10 s at 95 °C, followed by 50 cycles of 95 °C for 5 s, 60 °C or 65 °C for 34 s. SYBR Premix Ex Taq™ II (Takara Bio Inc.), 2 μL complementary (c)DNA sample as a template, and 0.4 μM of each gene-specific primer were reacted. Gene-specific primers for *GLN1;1*, *1;2*, *1;4*, *2*, and *ubiquitin*2 (*UBQ2*; GenBank J05508) were prepared following Ishiyama *et al*. (2004). *GLN1;3*-specific primers were *GLN1;3-RTF*(5′-TCC AAC CAA CAA GAG GCA CAA C-3′) and *GLN1;3-RTR* (5′-ACC AGA ACT AAT ACC CTC AAC A-3′). *GFP* specific primers were 204F (5′-AGT GCT TCA GCC GCT ACC C-3′) and 345R (5′-CCC TCG AAC TTC ACC TCG G-3′). Serial dilutions of plasmid were used as standards. Data were acquired and analyzed with the Light Cycler 480 Software version 1.2 (Roche Diagnostics K.K.). The dissociation curve confirmed a single PCR product. Water was used as a non-template control. The signal intensity was standardized to *UBQ2*. Three independent samples were quantified. Fold change in gene expression relative to that of the WT at 1 mM ammonium was determined on the basis of crossing points (CP) values (Pfaffl., 2001). RT-PCR primers for *GLN1;2*-specific primers were *Gln1;2RF and NK124* (5′-CGGATCATCCTTTCAA GGGTTCCAGAGGAG-3′), for *GLN1;3*-specific primers were *NK145* (5′-ATG TCTCTGCTCTCAGATCTCGTTA-3′) and *NK146* (5′-TCAACCGAGTATGGT CGTCTCAGCG-3′), and *UBQ2*-specific primers were prepared following Ishiyama *et al*. (2004).

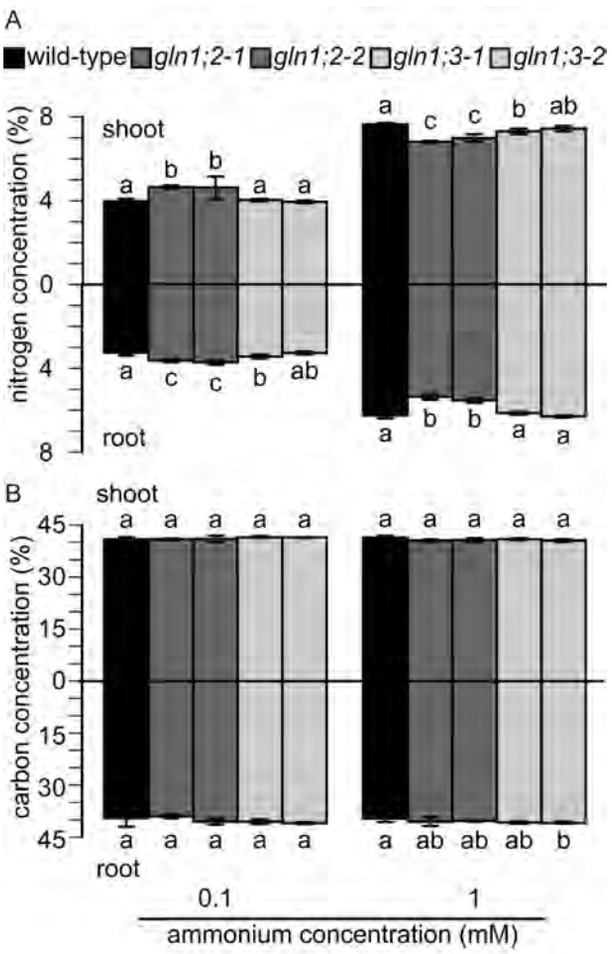


Figure 4.
Total nitrogen and carbon contents in roots and shoots of the wild-type (WT), *GLN1;2* and *GLN1;3* insertion lines
(A) Total nitrogen contents in roots and shoots. (B) Total carbon contents in roots and shoots of the WT (filled columns), *GLN1;2* insertion lines (dark gray columns) and *GLN1;3* insertion lines (light gray columns). Plants were grown hydroponically, supplemented with either 0.1 or 1 mM ammonium for 6 weeks. Bars indicate means ± standard deviation (SD) (n = 6). 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.

Isolation of T-DNA insertion lines for *GLN1;2* and *GLN1;3*

Arabidopsis (*Arabidopsis thaliana*) accession line Columbia (Col-0) was used as the WT. The following T-DNA insertion lines in the Col-0 genetic background were used:

gln1;2- 1 (At1g66200; SALK_145235), *gln1;2-2* (SALK_102291), *gln1;3-1* (At3g17820; SALK_002524), *gln1;3-2* (SALK_038156), and *gln1;3-3* (SALK_148604C). T-DNA insertion lines were obtained from SALK institute, self-fertilized, and selected for T-DNA homozygous plants. The T-DNA positions were determined by PCR using primers for T-DNA, T-*DNA LB-01* (5′-CCAGTAC ATTAAAAACGTCCGCAATGTGTT-3′) and *T-DNA RB-01* (5′GAATACAGTGAT CCGTGCCGCCCTG-3′); for the *GLN1;2* gene, *GLN1;2F* (5′-ATGAGTCTTCTT GCAGATCTTGTTA-3′) and *GLN1;2R* (5′TTTCAATAAAGGTCAAACAAAC AGA-3′); and for the *GLN1;3* gene, and *GLN1;3F* (5′-ATGTCTCTGCTCTCAGA TCTCGTTA-3′) and *GLN1;3R* (5′-TCAA CCGAGTATGGTCGTCTCAGCG-3′)

Two T-DNA insertion lines, *gln1;2-1* and *gln1;3-1*, were crossed, and the double insertion line, *gln1;2*:*gln1;3* was isolated.

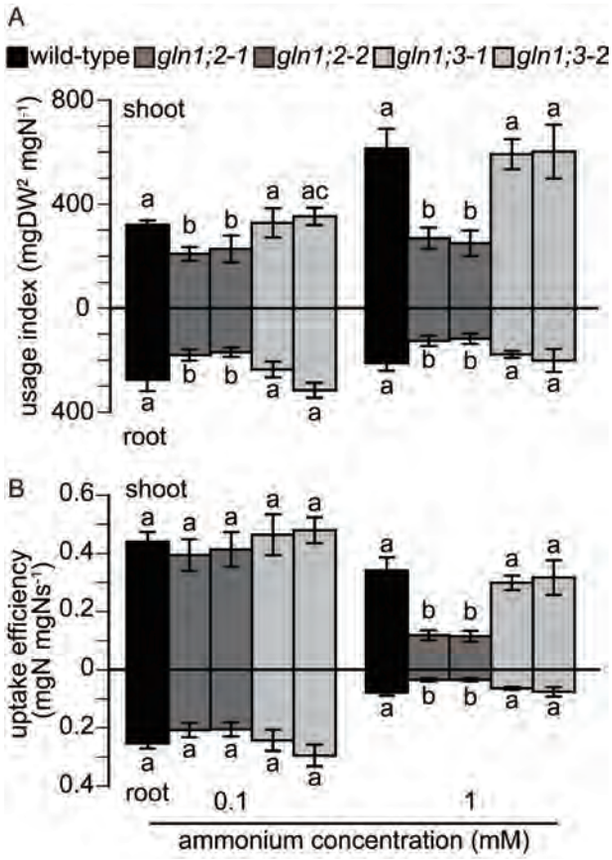


Figure 5.
Nutrient use efficiency in roots and shoots of the wild-type (WT) and *GLN1;2* and *GLN1;3* insertion lines
(A) Usage index was calculated based on the following formula:
UI = Sw × (Sw ÷ N)
where UI is usage index, Sw is shoot weight, and N is nitrogen in organs.
(B) Uptake efficiency was calculated based on the following formula:
UpE = Nt ÷ Ns
Where UpE is uptake efficiency, Nt is total nitrogen in the plant, and Ns is nitrogen supply (g per plant).
The values of the WT (filled columns), *GLN1;2* insertion lines (dark gray columns) and *GLN1;3* insertion lines (light gray columns) are shown. Plants were grown hydroponically for 6 weeks, supplemented with either 0.1 or 1.0 mM ammonium, and 10 μM nitrate. Bars indicate means ± standard deviation (SD) (n = 6). 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.

Nitrogen and carbon content

Plants were grown in a nutrient solution containing either 0.1 or 1 mM NH_4Cl as the major nitrogen source for 6 weeks. Plant samples were dried and powdered with a Tissue Lyser II (Qiagen, K. K.) at 20 Hz for at least 15 min. Samples were weighed with an ultra-microbalance (UMX2, Mettler Toledo International Inc., Tokyo, Japan) in tin capsules. Weights of samples were always between 1.000 and 1.050 mg. Nitrogen and carbon were determined with an elemental analyzer (Flash2000, Thermo Fisher Scientific K. K., Yokohama, Japan)

Uptake efficiency (UpE) and usage index (UI) (Good *et al*., 2004) were calculated to evaluate nutrient use efficiency in WT and *GLN1* insertion lines. UI is an index for the efficiency with which the N absorbed is utilized to produce biomass (Siddiqi and Glass, 1981). UpE is an index for the efficiency of uptake (Moll *et al*., 1982). Experiments were repeated at least twice with similar results, and representative values of one experiment are shown.

Free amino acids and ammonium measurement

Plant samples were frozen in liquid nitrogen and then milled with the Tissue Lyser II at 23 Hz for 1 min. Samples were suspended in 10 mM HCl and mixed in the Tissue Lyser II at 20 Hz for 2 min. After centrifugation at 20,500 g for 15 min at room temperature, the supernatant was transferred to an Amicon® Ultra 3K filter cup (Millipore, Bedford, MA) on a 2 mL tube and centrifuged again at 20,500 g for 30 min at room temperature. Amide residues of both amino acids and ammonium were labeled with the AccQ-Tag Ultra Derivatization Kit (Nihon Waters K. K., Tokyo, Japan), as previously

described (Konishi *et al*, 2014). Labeled samples were separated and analyzed on an ACQUITY Ultra Performance Liquid Chromatograph (UPLC) H-Class (Nihon Waters K. K.). Experiments were repeated at least twice with similar results and representative values of one experiment are shown.

Xylem sap preparation

Plants were hydroponically grown in nutrient solution (Loqué *et al*., 2006) for 42 days and transferred to a nutrient solution without nitrogen for 3 days. Plants were transferred again to the solution containing 0.1 or 3 mM NH_4Cl and 10 µM KNO_3 and the hypocotyls were excised with a razor (Feather Safety Razor Co., Ltd., Osaka, Japan) to collect xylem sap. Xylem sap was collected by harvesting the leaching solution from a cross-section at 24 h after plant transfer. The xylem sap was collected for 30 min after excision. Ammonium supply always started at 01:00 PM. Experiments were repeated at least twice with similar results, and representative values of one experiment are shown.

Statistics

All data sets were analyzed using the Microsoft Excel add-in software (Social Survey Research Information Co., Ltd., Tokyo, Japan).

Results

Dose-dependent effect of ammonium on *Arabidopsis* growth under low-nitrate conditions in hydroponic culture

In the present study, we evaluated the growth of *A. thaliana* Col-0 (WT) in a nutrient solution containing 10 µM KNO_3, and supplemented with 0.1, 0.3, 1, 2, 3, 5, and 10 mM NH_4Cl. Figure 1 shows the phenotype of the WT plants after 6 weeks in a hydroponic culture. As can be seen in Figure 1A, WT shoots showed maximal growth under 1 or 2 mM NH_4Cl.

However, the growth was decreased when the concentration of NH_4Cl in the nutrient solution was > 2 mM. The shoot and root dry weight of the WT plants tended to decrease at 3 mM, and was reduced by half at 5 mM compared with those at 1 mM. Growth was even more strongly inhibited at 10 mM.

GLN1;2 is the main isoform that assimilates ammonium over a wide range of growth ammonium concentrations

Two T-DNA insertion lines for *GLN1;2* (Fig. 2A) and those for *GLN1;3* (Fig. 2B) were isolated from Col. One of the T-DNA insertion lines, SALK_102291, was identical to that of the previous study (Lothier *et al*., 2011), whereas SALK_148604 was identical to that of Dragićević et al. (2014). qPCR analysis showed that *GLN1;2* mRNA was not detectable in *gln1;2-1*, whereas it was slightly expressed in *gln1;2-2* (Fig. 2E). Although qPCR showed slight expression, RT-PCR showed no visible *GLN1;2* expression in either T-DNA insertion line (data not shown). The expression of the other GS isozymes, *GLN1;1* (Fig. 2D), *GLN1;3* (Fig. 2F), *GLN1;4* (Fig. 2G), and *GLN2* (Fig. 2C) appeared unchanged in T-DNA insertion lines for *GLN1;2*. *GLN1;5* was not detectable (Fig. 2H) in roots.

Figure 3 shows the different contributions of *GLN1;2* and *GLN1;3* to ammonium nutrition. *GLN1;2* insertion lines showed marked reduction in dry weight compared to the WT. In addition, supplying ammonium led to a dose-dependent reduction in dry weight of *GLN1;2* insertion lines, whereas *GLN1;3* insertion lines showed no reduction. *GLN1;2* insertion lines showed a 60% reduction in dry weight at 1 mM ammonium, but only a 25% reduction at 0.1 mM (Fig. 3A and B). Conversely, there were no significant difference between WT and *gln1;2* in the nutrient solution containing either 1 or 10 mM nitrate (Fig. S2).

Figure 4 shows nitrogen and carbon concentrations in both shoots and roots of *GLN1* insertion lines. Carbon concentration in shoots and roots ranged from 35 to 40%, and there was no significant difference from the WT (Fig.

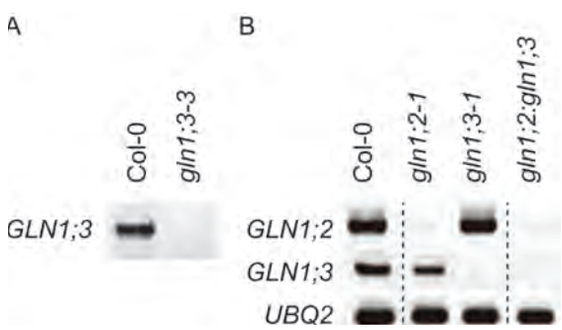
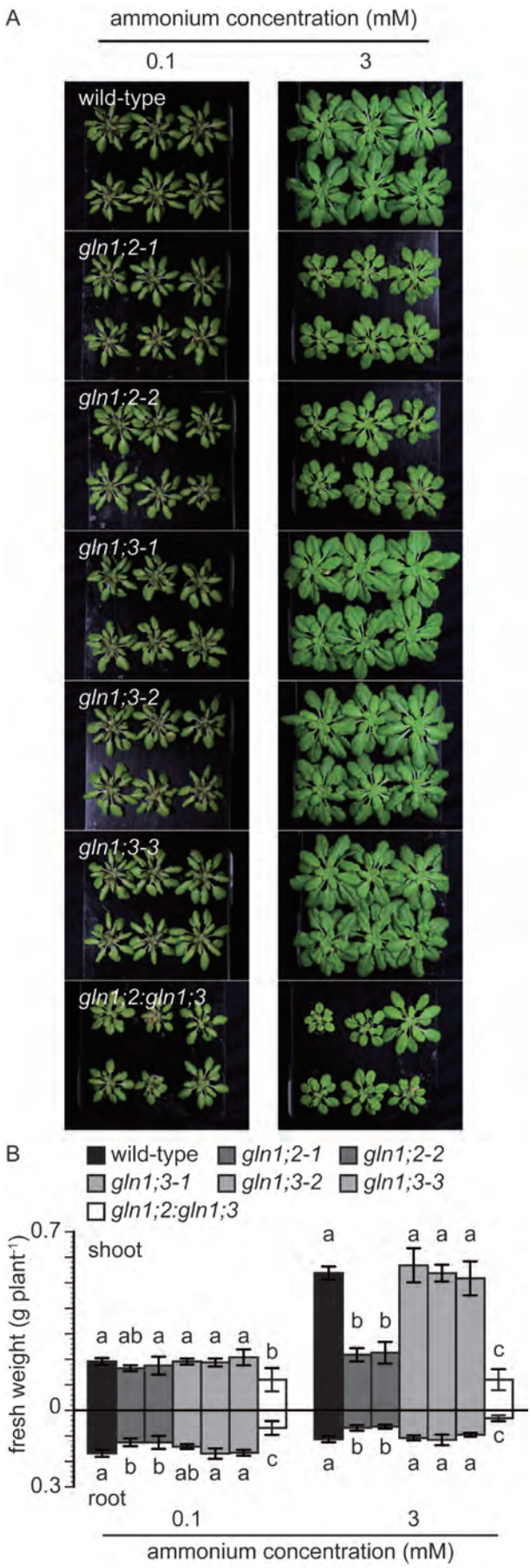


Figure 6.
Isolation of double insertion line for *GLN1;2* and *GLN1;3*
(A) Reverse transcription polymerase chain reaction (RT-PCR) analysis of root RNA from single insertion line for *GLN1;3*. The *GLN1;3* insertion line named *gln1;3-3* is identical to GLN1;3 KO in a previous study (Dragićević *et al*., 2014).
(B) RT-PCR analysis of root RNA from single insertion lines and their corresponding wild-type and from the double insertion line.
Plants were grown hydroponically for 6 weeks, supplemented with 0.1 mM ammonium and 10 µM nitrate as a major nitrogen source.



4B). The total nitrogen concentrations in the WT ranged from 3 to 4% at 0.1 mM ammonium condition, and increased to 6–8% under 1 mM ammonium (Fig. 4A). Nitrogen concentrations in *GLN1;2* insertion lines were significantly higher than those in the WT under 0.1 mM ammonium, and were lower under 1 mM (Fig. 4A). *GLN1;3* insertion lines showed no clear changes in nitrogen concentration under either 0.1 or 1 mM ammonium in comparison with the WT (Fig. 4A).

Figure 5 illustrates UI and UpE in *GLN1;2* and *GLN1;3* insertion lines with the WT. The effects of T-DNA insertion in *GLN1;2* on UI and UpE were dramatic (Fig. 5). *GLN1;2* insertion lines showed markedly reduced UI, especially under higher ammonium supply, whereas *GLN1;3* insertion lines did not show changes in UI under high or low ammonium supply in comparison with the WT (Fig. 5A). *GLN1;2* insertion reduced UI by 30% under 0.1 mM ammonium and by 50% under 1 mM (Fig. 5A) in comparison to the WT. UpE was reduced in *GLN1;2* insertion lines only at higher ammonium supply (Fig. 5B). *GLN1;2* insertion lines showed a 65% decrease in UpE under 1 mM ammonium supply (Fig. 5B). *GLN1;3* insertion did not change UpE under either high or low ammonium supply.

Figure 7.
Growth of the wild-type (WT) and *GLN1;2* and *GLN1;3* insertion lines under low nitrate supply, and the effect of ammonium supply in nutrient solution (A) Phenotype of the WT and insertion lines for *GLN1;2* and *GLN1;3*. (B) Shoot and root dry weights of the WT (filled columns), *GLN1;2* insertion lines (dark gray columns), *GLN1;3* insertion lines (light gray columns), and *GLN1;2:GLN1;3* double insertion line (opened column). Plants were grown for 6 weeks in nutrient solutions containing 0.1 or 3 mM ammonium and 10 µM nitrate as nitrogen source. Bars indicate means ± standard deviation (SD) ($n = 6$). 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.

To clarify the overlapping functions of GLN1;2 and GLN1;3, two *gln1* insertion lines, *gln1;2* and *gln1;3*, were crossed, and a double insertion line, *gln1;2*:*gln1;3* was isolated (Fig. 6). RT-PCR analysis indicated that double insertion line expressed neither *GLN1;2* nor *GLN1;3* (Fig. 6B). In Figure 7, statistical analysis of fresh weight is presented. In the single *gln1;2*, the fresh weight was decreased by half under 3 mM ammonium condition, whereas in the single *gln1;3*, it was not much different from wild-type (Fig. 7). The fresh weight of *gln1;2*:*gln1;3* was significantly different from single insertion lines. Under 0.1 mM ammonium condition, it was decreased by 36% compared with *gln1;2*, and decreased by 46% compared with *gln1;3*. Under 3 mM ammonium condition, it showed 48% and 77% reduction, respectively (Fig. 7).

To distinguish the functions of two root GS1 isozymes in *Arabidopsis* in ammonium assimilation, the free amino acid and ammonium concentrations were compared between *GLN1* insertion lines and WT under 0.1 and 3 mM ammonium supply. Figure 8 summarizes the changes in free ammonium and glutamine in the WT and *GLN1* insertion lines. Ammonium concentration was sharply increased in *GLN1;2* insertion lines (Fig. 8C), whereas glutamine (Fig. 8B) as well as total amino acid (Fig. 8A) concentrations were decreased. Supplementary Figure S3 shows amino acid composition in shoot and root of the WT and insertion lines. Glutamine accounted for >40% of total amino acids in shoot and >70% in root at 3 mM ammonium. Serine, asparagine, and arginine accounted for approximately 10% of total amino acids in shoot at 3 mM ammonium. A loss of GLN1;2 led to a decrease in glutamine ratio but an increase of serine ratio in the whole plant at 3 mM ammonium. Aspartate, threonine, and alanine ratios were increased in *GLN1;2* insertion lines.

Given that the rice *GS1;2* mutant showed increased ammonium and decreased glutamine (Funayama *et al.*, 2013), we investigated the changes of those nitrogen compounds in xylem exudates from *Arabidopsis GLN1;2* and *GLN1;3* insertion lines after supplying ammonium (Fig. 9). Figure 9 illustrates the changes in glutamine and ammonium concentrations in xylem sap over 24 h after supplying ammonium. *GLN1;2* insertion lines resulted in a 50% decrease

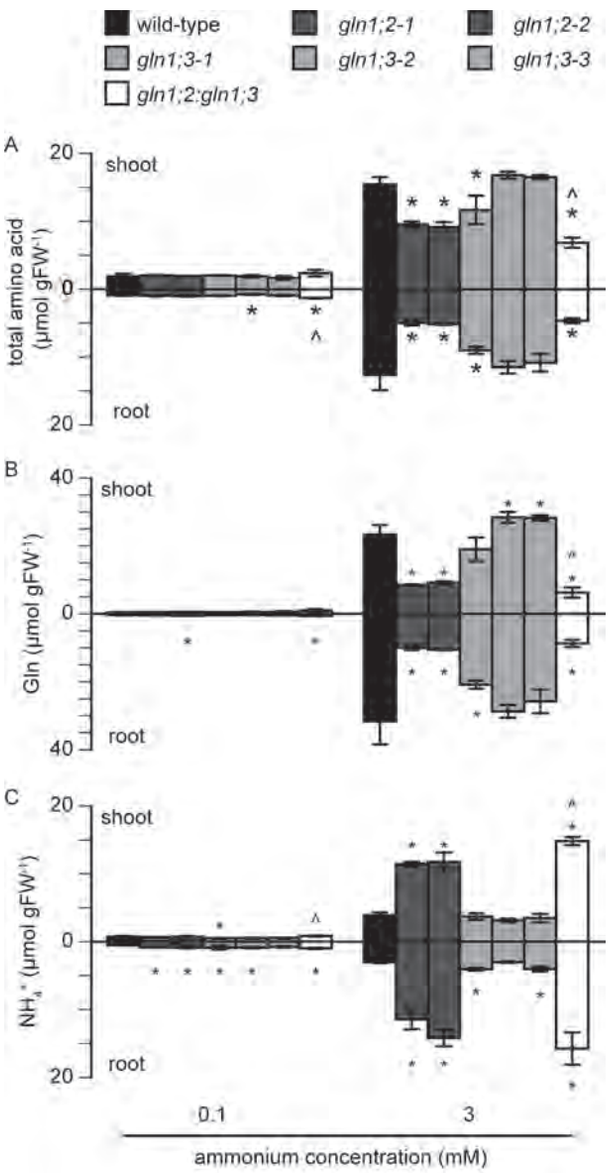


Figure 8.
Ammonium accumulation and amino acid reduction in *GLN1;2* and *GLN1;3* insertion lines under ammonium supply
The concentrations of total free amino acids (A), free glutamine (B), and free ammonium (C) were measured in roots (lower panel) and shoots (upper panel) of the wild-type (WT) (filled columns), *GLN1;2* insertion lines (dark gray columns), *GLN1;3* insertion lines (light gray columns) and *GLN1;2:GLN1;3* double insertion line (opened column). Plants were grown hydroponically for 6 weeks, supplemented with 0.1 or 3 mM ammonium and 10 µM nitrate as a major nitrogen source. Bars indicate means ± standard deviation (SD) (n = 3). One-way analysis of variance (ANOVA) followed by Dunnett tests was used, and significant differences at $p < 0.05$ between WT and *GLN1;2* or *GLN1;3* insertion lines are indicated with an asterisks (*) and between *GLN1;2* insertion lines and *GLN1;2:GLN1;3* double insertion line is indicated with a circumflex (^).

in glutamine in comparison to the WT (Fig. 9A). The ammonium concentration was higher in *GLN1;2* than in the WT (Fig. 9B).

GLN1;2 absence reveals a function for GLN1;3 under ammonium nutrition

GLN1;3 insertion lines did not show reduced dry weight under any conditions tested except 0.5 mM ammonium supply (Fig. 3 A and 3B). Root dry weight was decreased by 20–30% in *GLN1;3* insertion lines under 0.5 mM ammonium (Fig. 3B). Since variability was observed among plants (Fig. 3), the third insertion line, *gln1;3-3*, was used in further analysis (Fig. 6). RT-PCR showed no detectable signal for *GLN1;3* in the *gln1;3-3* insertion line (Fig. 6). Given that no significant difference between WT and *GLN1;3* insertion lines was observed (Figs. 3–5, 7, 9 and, S2), *gln1;2* and *gln1;2*:*gln1;3* double insertion line (Fig. 6) were compared in 0.1 and 3 mM ammonium (Fig. 7).

GLN1;3 insertion lines showed no significant decrease in fresh weight under the tested condition (Fig. 7). Compared with *gln1;2*, the root fresh weight was decreased by half and the shoot dry weight was decreased 30%–45% in *gln1;2*:*gln1;3* (Fig. 7).

Given that *gln1;2*:*gln1;3* showed decreased biomass, free amino acids and ammonium were measured at the 0.1 and 3 mM ammonium conditions (Fig. 8). *GLN1;3* insertion lines showed no clear changes in ammonium concentration (Fig. 8C). No significant differences were observed in the concentrations of total amino acid (Fig. 8A) and glutamine (Fig. 8B) between *gln1;3* and the WT. The total amino acid and glutamine in *gln1;2*:*gln1;3* was lower than that in *gln1;2* shoot (Fig. 8A and 8B), whereas ammonium in double insertion line was higher than *gln1;2* (FIg. 8C). Supplementary Figure S3 shows that a loss of GLN1;3 did not dramatically change the amino acid composition.

Xylem sap analysis indicated that the glutamine concentration in *gln1;2*:*gln1;3* was significantly lower than that in *gln1;2* (Fig. 9A), whereas there was no significant difference in ammonium concentration (Fig. 9B). Under all conditions tested, *GLN1;3* insertion lines showed no statistical differences from the WT (Fig. 9).

The promoter activities of *GLN1;2* are enhanced in epidermis and cortex cell layers, and *GLN1;3* is constitutively localized in pericycle

Figure 10A summarizes the expression of *GLN* genes in *Arabidopsis* roots under 0.1, 1 and 3 mM ammonium supply. *Arabidopsis* roots highly accumulated *GLN1;2* under both high and low ammonium supply. Other *GLN* genes, *GLN1;1*, *GLN1;3*, *GLN1;4*, and *GLN2*, were all more highly expressed at 0.1 mM ammonium than under higher-ammonium

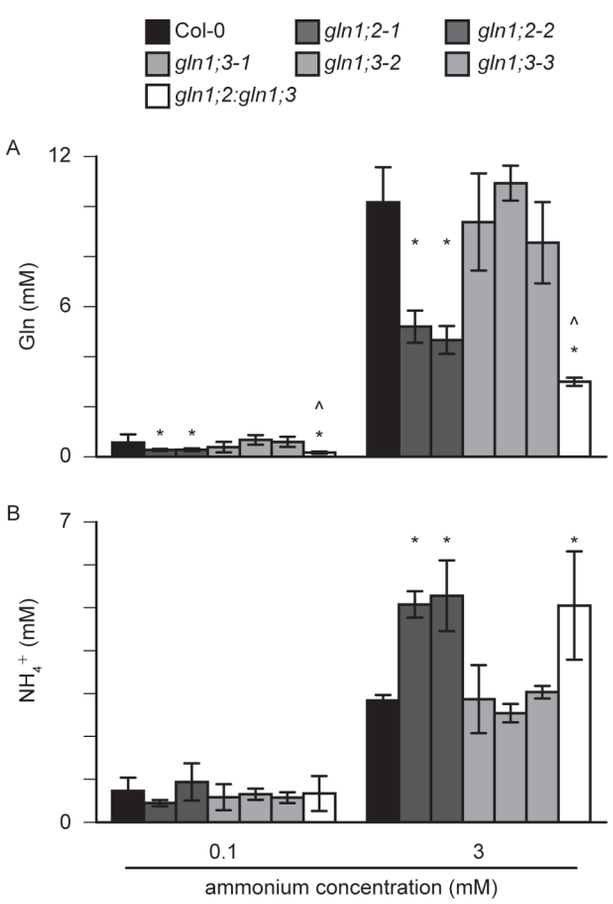


Figure 9.
Ammonium accumulation and glutamine reduction in xylem sap of insertion lines after ammonium was supplied
(A) The concentration of glutamine in xylem sap of the wild-type (WT) and transfer DNA (T-DNA) insertion lines for *GLN1;2* and *GLN1;3*. (B) The concentration of ammonium in xylem sap of the WT (filled columns), *GLN1;2* insertion lines (dark gray columns) and *GLN1;3* insertion lines (light gray columns), and *GLN1;2:GLN1;3* double insertion line (opened column). Plants were grown for 42 days in nutrient solution containing 2 mM ammonium nitrate and transferred to nutrient solution without nitrogen. After 3 days, the plants were again transferred to a nutrient solution containing either 0.1 or 3 mM ammonium and 10 μM nitrate. After 24 h, plants were excised and xylem sap was collected. Bars indicate means ± standard deviation (SD) (n = 4). One-way analysis of variance (ANOVA) followed by Dunnett tests were used, and significant differences at $p < 0.05$ between WT and *GLN1;2* or *GLN1;3* insertion lines are indicated with an asterisks (*) and between *GLN1;2* insertion lines and *GLN1;2:GLN1;3* double insertion line is indicated with a circumflex (^).

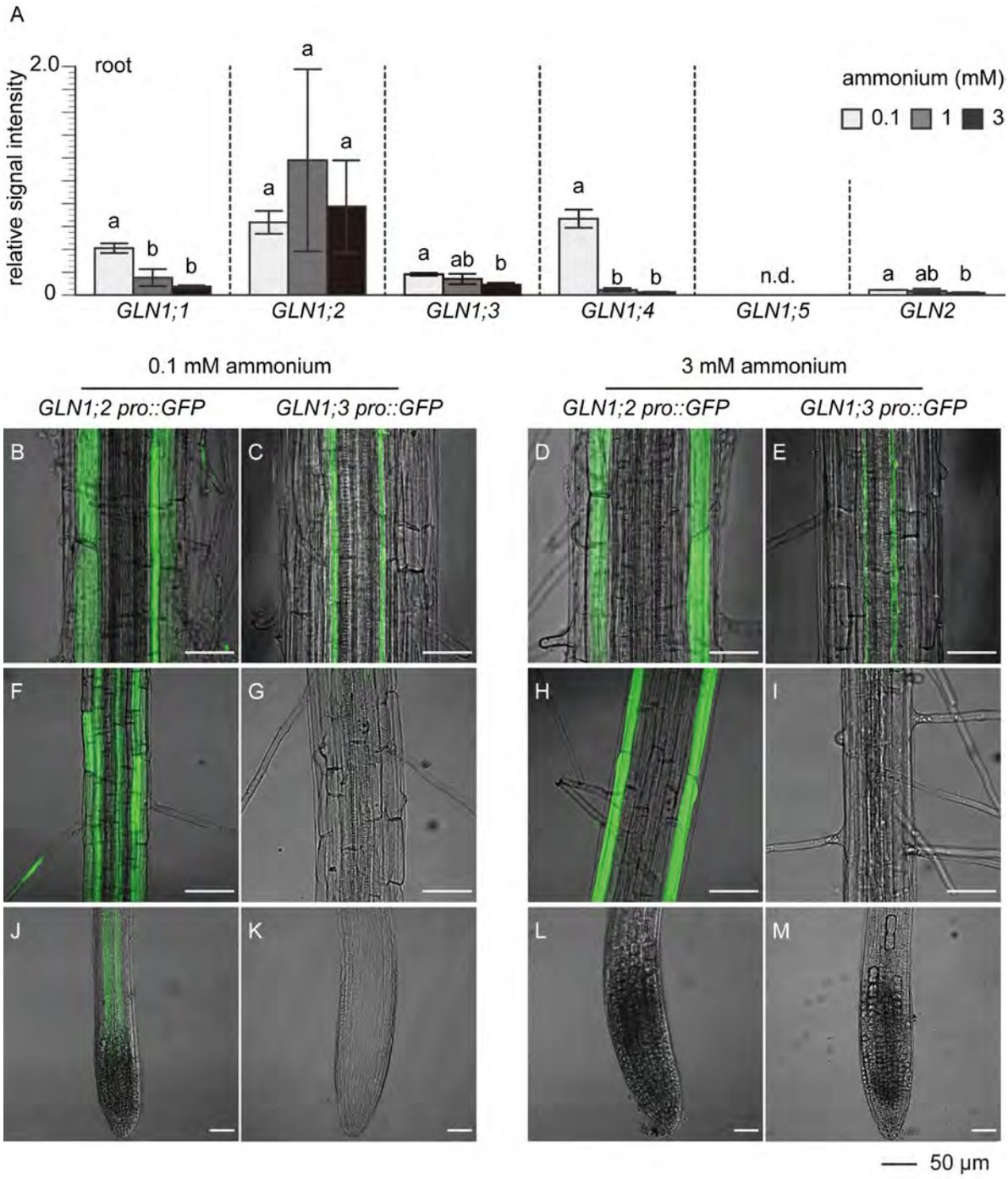
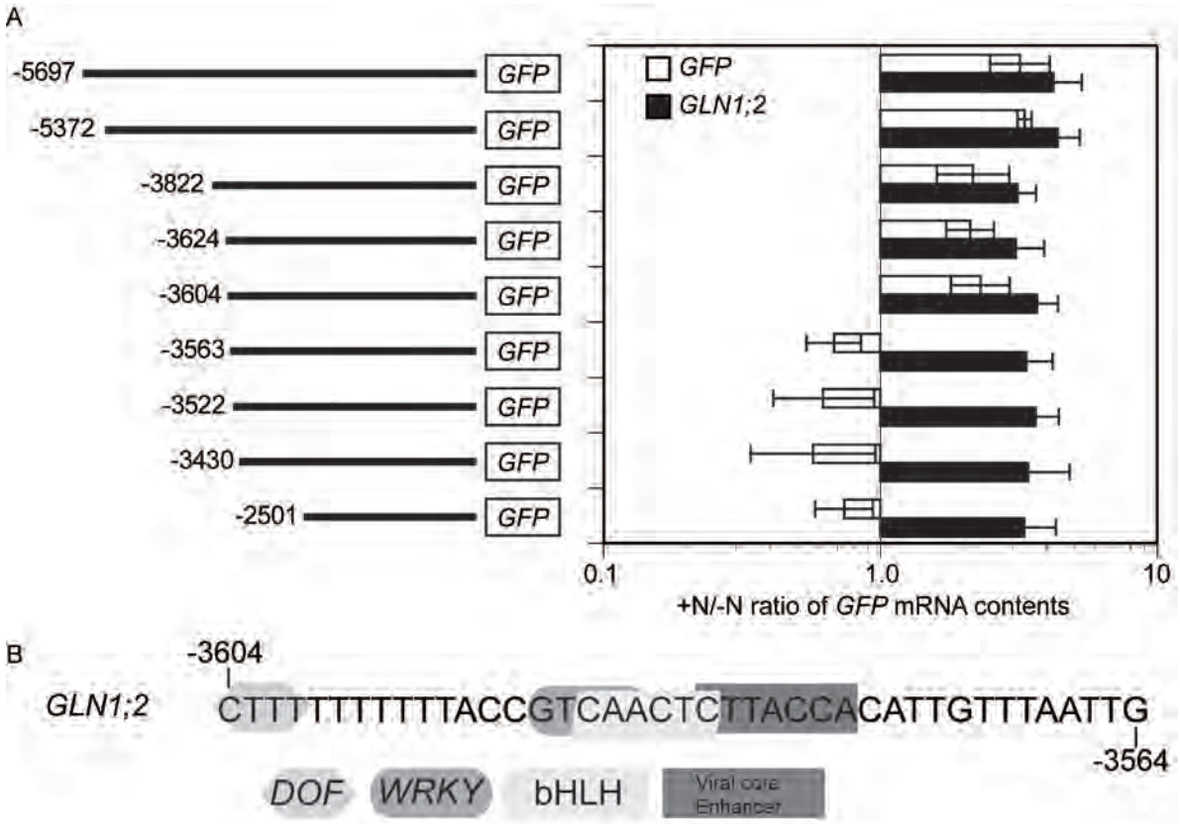


Figure 10.
Organ and cell type-specific expression of *GLN1* genes in *Arabidopsis* roots
(A) Quantitative real-time polymerase chain reaction (qPCR) analysis of root RNA from wild-type (WT) using gene-specific primers for *GLN1;1*, *GLN1;2*, *GLN1;3*, *GLN1;4*, *GLN1;5* and *GLN2*. Plants were grown in nutrient solutions containing either 0.1 (opened column) or 1 mM (gray column) or 3 mM (filled column) ammonium and 10 µM nitrate for 6 weeks. Ubiquitin2 (*UBQ2*) was used to standardize the signal intensity. Bars indicate means ± standard deviation (SD) (n = 3). Bars indicate means ± SD (n = 4). 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. (B–M) Localization of the promoter activities of *GLN1;2* (B, D, F, H, J, and L) and *GLN1;3* (C, E, G, I, K, and M). Transgenic plants expressing either *GLN1;2 promoter:GFP* or *GLN1:3 promoter:GFP* fusion gene constructs were grown for 6 weeks in nutrient solutions containing 0.1 (B, C, F, G, J and K) or 3 (D, E, H, I, L and M) mM ammonium and 10 µM nitrate as nitrogen source. Whole-mount images from root tips (J, K, L, and M), root hair zones (F, G, H, and I), mature parts (B, C, D, and E) were acquired by confocal laser scanning microscopy. Bars represent 50 µm.



conditions (Fig. 10A). *GLN1;2* accounted for only 34% of total *GLN* transcripts at 0.1 mM ammonium supply, but for almost 80% at 1 and 3 mM (Fig. 10A). *GLN1;5* was not detectable (Fig. 10A).

Figure 10B–M illustrates the localization of *GLN1;2* and *GLN1;3* promoter activity under a 0.1 or 3 mM ammonium condition. *GLN1;2* promoter activity was mainly localized in the epidermis and cortex (Fig. 10B, 10D, 10F, and 10H), whereas *GLN1;3*-dependent GFP was localized mainly in the pericycle of mature roots (Fig. 10C and 10E). However, *GLN1;3* promoter activity was localized in neither the root hair zone (Fig. 10G and 10I), nor root tips (Fig. 10K and 10M). Variable ammonium concentrations did not change the localization of *GLN1;3* promoter activity (Fig. 10). On vertical agar culture, ammonium supply highly induced *GLN1;2* promoter activity in the rhizosphere, whereas it did not change *GLN1;3* promoter activity (Fig. S4).

To identify the regulatory region for ammonium response of *GLN1;2* gene expression, we compared the responses of truncated versions of *GLN1;2* promoter-GFP constructs in transgenic *Arabidopsis* plants (Fig. 11 and Fig. S5). The full-length promoter, containing a genomic region 5,697 bp upstream of *GLN1;2* translational start codon, responded to ammonium in the medium and led to a significant increase in *GFP* mRNA accumulation (Fig. 11A). Quantitative real-time RT-PCR revealed that this full-length promoter could drive *GFP* expression on ammonium supply, cumulating the *GFP* levels up to three-fold those at the control nitrogen-starved condition (Fig. 11A and Fig. S5). The induction of *GFP* accumulation, driven by this full-length promoter, was consistent with increased the accumulation of *GLN1;2*. Following the 5′-deletion series of *GLN1;2* promoter-GFP constructs, there was no great difference in the fold-change induction of *GFP* expression as far as the position −3,604. However, the truncation of the promoter to −3,563 drastically reduced the *GFP* expression

Figure 11.
Deletion analysis of ammonium responsive for *GLN1;2* promoter in roots (A) 5′ deletion analyses between −5697 and −2501 of *GLN1;2* promoter were performed. Green fluorescent protein (*GFP*) was quantified in each transgenic plant root with real time polymerase chain reaction (qPCR) using specific primers. At least five independent lines of T2 transformants from each construct were grown on MGRL mediums for 2 weeks, and then after 2 weeks, plants were subjected to nitrogen starvation for 3 days prior to the treatment, and then transferred to the modified MGRL mediums without nitrogen or with 10 mM ammonium chloride. Means of five to ten independent samples and the standard deviations are indicated. Significant differences were identified using Student's t-test and are indicated using an asterisk (*) symbol.
(B) There are four predicted motifs for binding to Dof (−3,604 CTTT −3,601), WRKY proteins (−3,590 GTCAA −3,586), bHLH (−3,588 CAACTC −3,583), and viral core enhancer (−3,583 CTTACCA −3,577) in the 41 bp region.

(Fig. 11A). Nevertheless, the endogenous *GLN1;2* responded to the ammonium supply.

Discussion

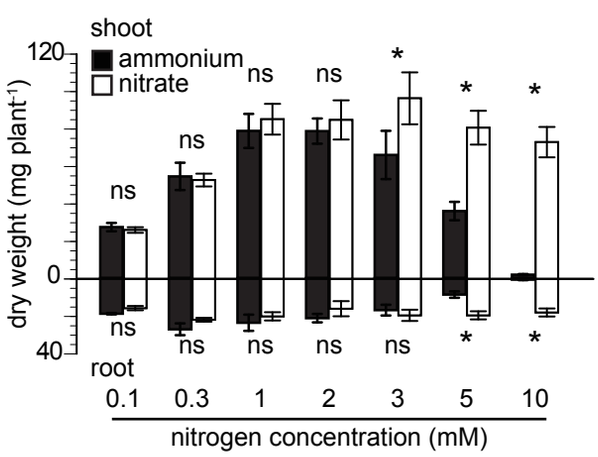
Earlier studies showed that a small amount of supplied nitrate (Garnica *et al*., 2010; Krouk *et al*., 2006; Yuan et al., 2007) or pre-culture in nitrate medium (Hachiya *et al*., 2012; Sarasketa *et al*., 2014) alleviated ammonium toxicity. Supplemented with a small amount of nitrate, the present study showed that ammonium toxicity appeared at 3 mM in hydroponic culture and that nitrogen deficiency appeared at 0.3 mM (Fig. 1). It is evident that the optimal ammonium concentration in nutrient solution is 1 or 2 mM. The phenotypes observed below 3 mM ammonium are related to general ammonium assimilation but not ammonium toxicity (Fig. S1).

Three independent T-DNA insertion lines for *GLN1;3* and *GLN1;2* (Fig. 2) and double insertion line for *GLN1;2* and *GLN1;3* were isolated (Fig. 6). The growth of insertion lines was compared with that of the WT in hydroponic culture (Figs. 1, 3, and 7). The contribution of *GLN1;3* to ammonium assimilation was not major in comparison with that of *GLN1;2* (Figs. 3 and 7). The comparison of *gln1;2*:*gln1;3* with *gln1;2* indicated the small but significant contribution of GLN1;3 to ammonium assimilation in roots (Figs. 6–9). GLN1;3 revealed its function only when GLN1;2 was not functional.

GLN1;3 promoter activity was localized to the pericycle and was independent of the external ammonium concentration (Fig. 9). In the root, the pericycle is required for xylem loading and for lateral root initiation (Beeckman *et al*., 2014). The pericycle associated GLN1;3 might be involved in xylem loading of glutamine. Indeed, xylem sap glutamine in *gln1;2*:*gln1;3* was significantly lower than that in *gln1;2* (Fig. 9), suggesting that the loading of glutamine to xylem was partly dependent on GLN1;3. The growth and localization might suggest that GLN1;3 assimilates concentrated symplastic ammonium around the stele. These findings extend the function of GLN1;3 from enzymatic characteristics to physiological functions in plant. The previous article showed that ammonium supply triggers lateral root development (Lima *et al*., 2010). Future work should focus on the contribution of pericycle localized GLN1;3 to root system architecture under ammonium supply.

It is likely that the difference in spatial and temporal expression of *GLN1;2* and *GLN1;3* determines the different responses of these two *GLN1* insertion lines to various ammonium concentrations. However, the post-translational regulation of the two GS1 isozymes *in planta* remains unknown. Growth analysis of transgenic plants expressing *GLN1;3* driven by the *GLN1;2* promoter in a *GLN1;2* and *GLN1;3* double-insertion line may be a promising approach.

Previous studies localized *GLN1;2* promoter activity in root vascular tissues (Guan *et al*., 2015; Ishiyama *et al*., 2004; Lothier *et al*., 2011). In the present study, *GLN1;2* promoter was longer than that in previous studies because shorter *GLN1;2* promoter (Ishiyama *et al*., 2004) did not respond to the ammonium supply (Fig. 10). The longer *GLN1;2* promoter-GFP shows the localization of GLN1;2 in the epidermis and cortex in ammonium supply (Figs. 10, 11, and S4). The promoter deletion analysis suggested that at least the sequences between −3,604 and −3,563 bp are necessary to enhance *GLN1;2* transcriptional activity in response to ammonium supply in root. A database search on Plant cis-acting regulatory DNA elements (Higo *et al*., 1999) showed that this region could be recognized by four-types of transcriptional factors (Fig. 11B) which are DNA-binding with one finger (DOF) (Yanagisawa 1996), WRKY, bHLH, and viral core enhancer. This result is in good agreement with previous articles suggesting DOF dependent nitrogen metabolism (Yanagisawa *et al*., 2004) and DOF dependent *GLN* expression (Rueda-López *et al*., 2008). *GLN1;2* accumulation



Supplementary Figure S1.
Growth of wild-type (WT) under various concentrations of nitrate or ammonium
(A) Growth of the WT in hydroponic solutions containing 0.1, 0.3, 1, 2, 3, 5, or 10 mM of either NH_4Cl or KNO_3 as the major nitrogen source, supplemented with 10 μM nitrate for 6 weeks. (B) Shoot and root dry weight of the same plants as in A. Bars indicate means ± standard deviation (SD) (n = 4-6). Significant differences at $*p < 0.05$ is indicated using an asterisk (*). Differences were analyzed using Student's t test.

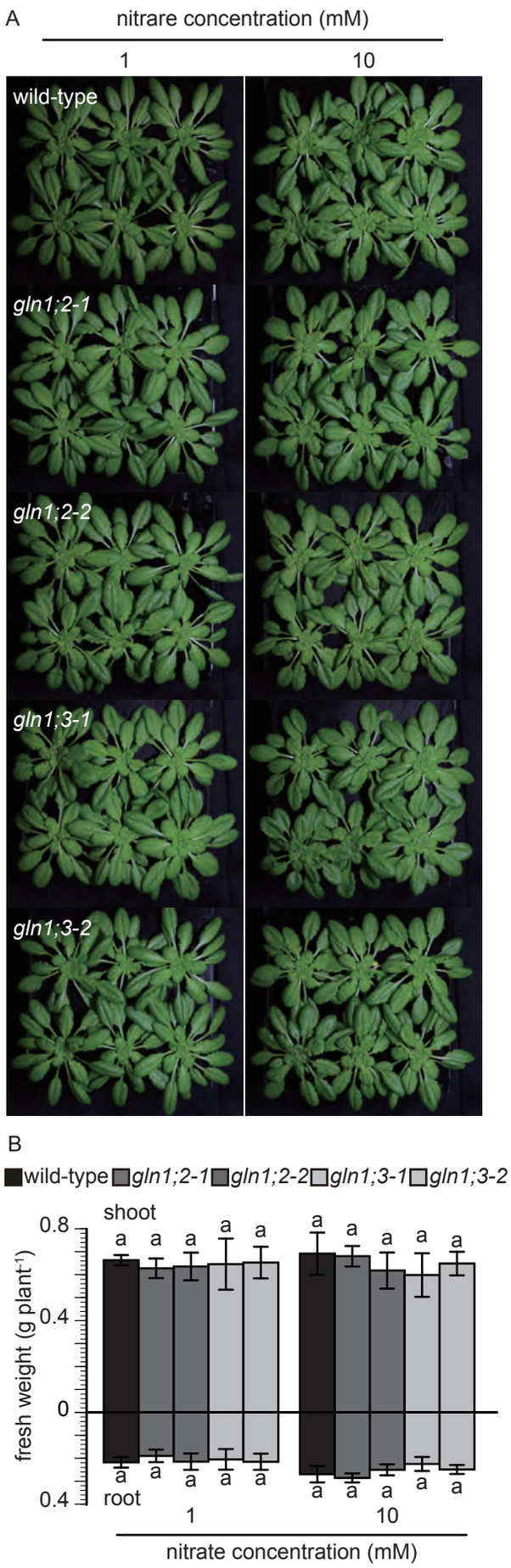


Fig. S2 Konishi et al

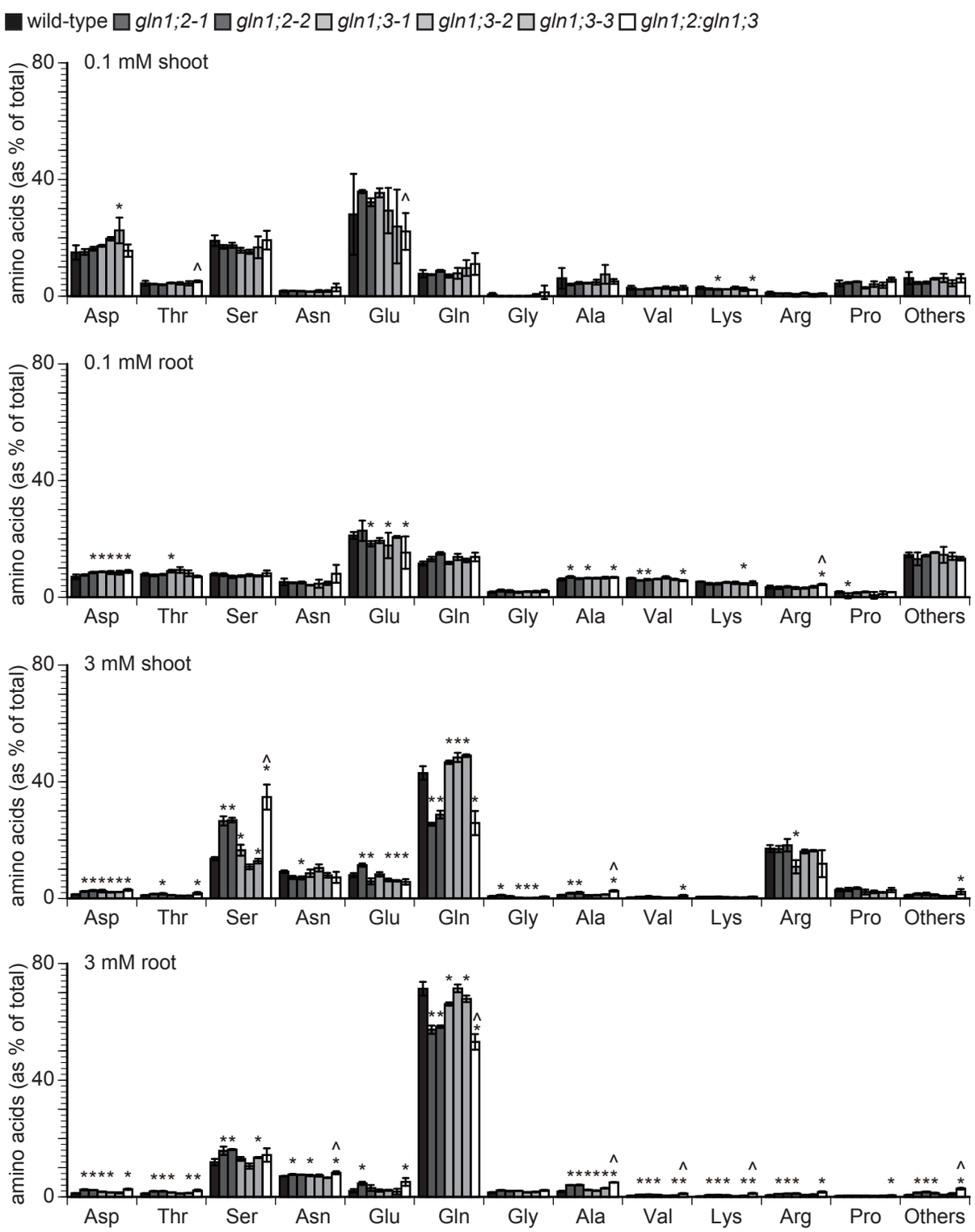
in response to the ammonium supply initially occurs in the epidermis cell layers of *Arabidopsis* roots, where this enzyme would have major metabolic functions in assimilating the ammonium uptake from the rhizosphere.

In addition to localization studies, a reverse-genetic analysis also suggested the importance of GLN1;2 in ammonium assimilation in *Arabidopsis* (Guan *et al.*, 2016; Lothier *et al.*, 2011). Because the Casparian strip blocks apoplastic ammonium transport between pericycle cells and the soil solution (Loqué *et al.*, 2006), most apoplastic and symplastic ammonium should be assimilated by GLN1;2. GLN1;2 contributed to ammonium assimilation not only at a higher concentration of 2–20 mM, as shown in previous articles (Guan *et al.*, 2016; Lothier *et al.*, 2011) but also at a lower concentration of 0.3 mM (Fig. 3). Because ammonium in soil solution varies from 0.1 to 0.8 (Miller *et al.*, 2007), the presence of such broad GLN1;2 contribution is a realistic finding.

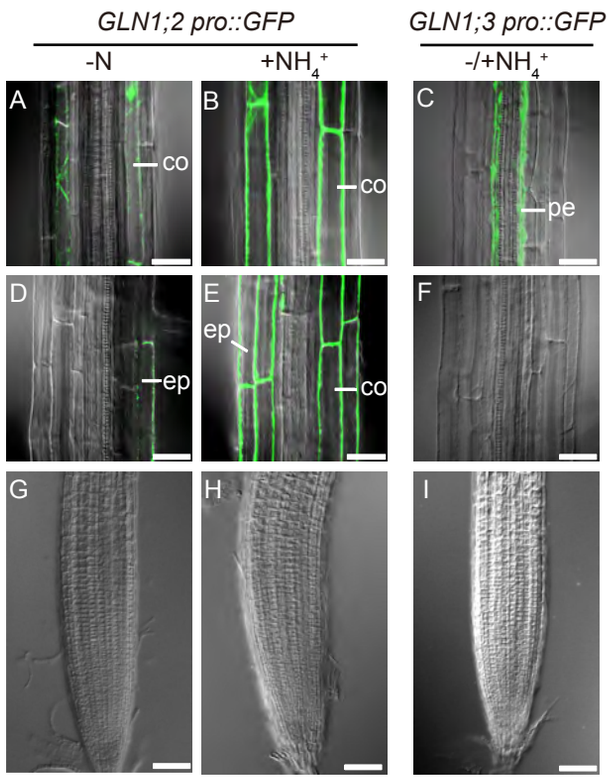
Ammonium supply increased the proportion of *GLN1;2* in the total *GLN* isogene pool (Fig. 10). This result was consistent with results obtained in agar culture (Ishiyama *et al.*, 2004). Given that *GLN1;5* appears to be a pollen-specific GS1 (Schmid *et al.*, 2005; Soto *et al.*, 2010) and that it was not detectable in

Supplementary Figure S2.
Growth of wild-type (WT) and insertion lines under low and high concentrations of nitrate

(A) Growth of the WT (filled column), *GLN1;2* insertion lines (dark gray column), and *GLN1;3* insertion lines (light gray column) in hydroponic solutions containing either 1, or 10 mM KNO_3 as the major nitrogen source, supplemented with 10 μM nitrate for 6 weeks. (B) Shoot and root dry weight of the same plants as in A. Bars indicate means ± standard deviation (SD) (n = 6). 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.



Supplementary Figure S3.
Amino acid composition in shoot and root of *GLN1;2* and *GLN1;3* insertion lines at 0.1 and 3 mM ammonium supply.
The concentrations of individual amino acid (as % of total) in shoot and root of the wild-type (WT) (filled columns), *GLN1;2* insertion lines (dark gray columns) and *GLN1;3* insertion lines (light gray columns), and *GLN1;2:GLN1;3* double insertion line (opened column) grown at either 0.1 or 3 mM ammonium were determined. Plants were grown hydroponically for 6 weeks, supplemented with 0.1 or 3 mM ammonium and 10 µM nitrate as a major nitrogen source. Bars indicate means ± standard deviation (SD) (n = 3). One-way analysis of variance (ANOVA) followed by Dunnett tests were used, and significant differences at $p < 0.05$ between WT and *GLN1;2* or *GLN1;3* insertion lines are indicated using a asterisks (*), and between *GLN1;2* insertion lines and *GLN1;2:GLN1;3* double insertion line are indicated as a circumflex (^).



Supplementary Figure S4.
Localization of the promoter activities of *GLN1;2* and *GLN1;3* on vertical agar culture.
Localization of the promoter activities of *GLN1;2* and *GLN1;3*. Transgenic plants expressing either *GLN1;2 promoter:GFP* (A, B, D, E, G and H) or *GLN1:3 promoter:GFP* (C, F and I) fusion gene constructs were grown on agar plates containing 7 mM nitrate as a nitrogen source for 14 days and transferred to the plates containing no nitrogen. After 3 days, the plants were again transferred to the plates containing either ammonium supplemented ($+NH_4^+$) or ammonium deficient ($-NH_4^+$). Whole-mount images from root tips (G, H, and I), root hair zones (D, E, and F) and mature parts (A, B, and C) were taken with confocal laser scanning microscopy after 24 h. co, ep and pe indicate cortex cell, epidermal cell, and pericycle cell, respectively. Bars represent 100 μm.

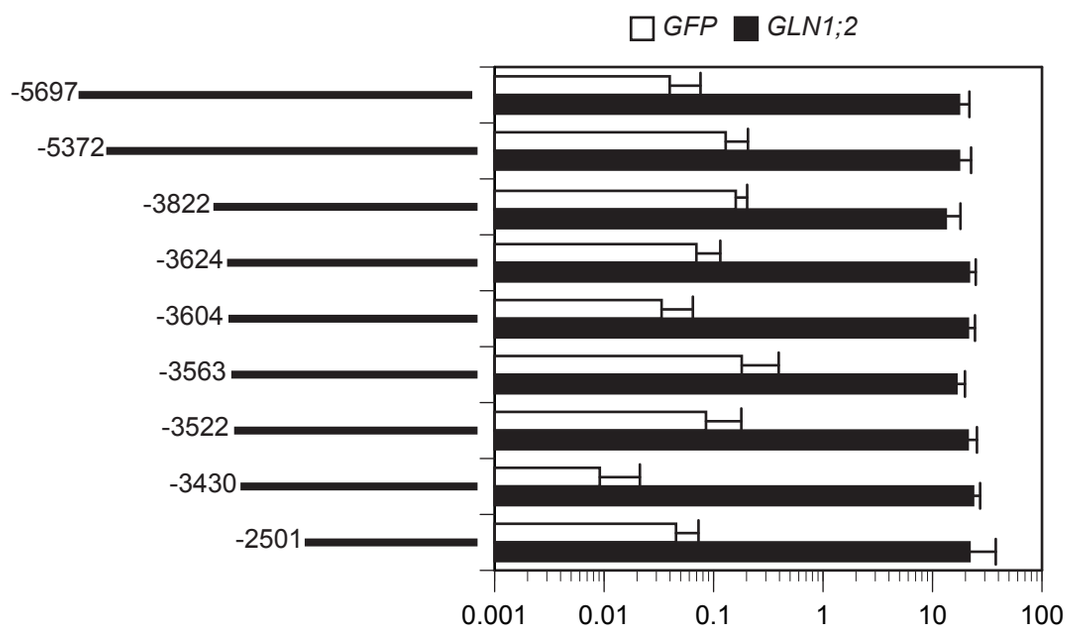
roots (Figs. 2 and 10), the five *GLN* genes may reflect the population of root *GLN*. Increasing the ammonium concentration severely inhibiting the growth of *gln1;2* (Figs. 3 and 7). Inhibition of both nitrogen use and nitrogen acquisition (Fig. 5) resulted in reduced nitrogen concentration (Fig. 4). These results are partially consistent with results of previous studies (Lothier *et al*., 2011). In addition to those phenotypes, *GLN1;2* insertion dramatically increased free ammonium concentration not only in plant organs but also in xylem exudate, whereas free glutamine concentration was decreased (Figs. 8, 9, and S3). Xylem sap analysis indicated that GLN1;2 dependent ammonium assimilation mainly occurred at roots when ammonium concentration was <3 mM. Excess ammonium supply appears to saturate the capacity of root GLN1;2; therefore, shoot GLN1;2 is essential for overcoming ammonium toxicity (Guan *et al*., 2016).

It is already known that ammonium supply triggers the accumulation of glutamine (Clark, 1936). Amino acid composition analysis showed that arginine accounts for approximately 15% at 3 mM ammonium in shoot (Fig. S3), whereas arginine accounts for only <1% in nitrate-grown plants (Lothier *et al*., 2011). Due to the highest nitrogen to carbon ratio among the 21 proteinogenic amino acids, arginine is a major storage for organic nitrogen in plants (Winter *et al*., 2015). Accumulated glutamine appears to be converted to arginine in shoot.

Neither *GLN1;2* nor *GLN1;3* insertion lines showed statistically different growth in either 1 mM or 10 mM nitrate supply in the present study. This result is not consistent with previous work (Lothier *et al*., 2011), showing the biomass reduction in rosette leaves of *GLN1;2* insertion line when the plant was grown in 10 mM as sole nitrate condition, whereas there was no difference in the 2 mM

condition. The reason for the mismatches of the two studies could be explained by different cultural conditions and genetic backgrounds used. There were differences in temperature, light, and nutrient (besides nitrate) concentration. A previous article used *GLN1;2* insertion line in Ws as a genetic background , whereas conversely, the present work used Col. The growth of Ws and Col showed differences under a nitrate supplied condition (Lothier *et al*., 2011).

In conclusion, the contribution of GLN1;2, an ammonium-inducible GLN1, to ammonium assimilation was much higher than that of GLN1;3. GLN1;3 may assimilate the ammonium that was not assimilated by GLN1;2. Although the present study provides insight into the physiological functions of GLN1;2 and GLN1;3, they are not the only GLN1 isozymes expressed in the *Arabidopsis* root. It will be necessary to investigate the functions of GLN1;1 and GLN1;4 and high-affinity to ammonium to elucidate the full set of ammonium-assimilatory mechanisms in *Arabidopsis* plants.



Supplementary Figure S5.
The promoter activity absolute data at nitrogen deficient condition.
5′ deletion analyses between −5697 and −2501 of *GLN1;2* promoter were performed. Green fluorescent protein (*GFP*) and GLN1;2 were quantified in each transgenic plant root with real time polymerase chain reaction (qPCR) using specific primers. At least five independent lines of T2 transformants from each construct were grown on MGRL mediums for 2 weeks, and then after 2 weeks, plants were subjected to nitrogen starvation for 3 days prior to the treatment, and then transferred to the modified MGRL mediums without nitrogen. Means of five to ten independent samples and the standard deviations are indicated.

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Supplementary data

Supplementary Fig. S1.

Growth of the WT under various concentrations of either nitrate or ammonium.

Supplementary Fig. S2.

Growth of the WT and T-DNA insertion lines for *GLN1;2* and *GLN1;3* under 1 or 10 mM nitrate supply.

Supplementary Fig. S3.

Amino acid composition in WT and T-DNA insertion lines for *GLN1;2* and *GLN1;3* under 0.1 or 3 mM ammonium supply.

Supplementary Fig. S4.

Localization of the promoter activities of *GLN1;2* and *GLN1;3* on vertical agar culture.

Supplementary Fig. S5.

Localization of the promoter activities of *GLN1;2* and *GLN1;3* on vertical agar culture.