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

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Abstract

Glutamine synthetase (GS) catalyzes a reaction that incorporates ammonium into glutamate and yields glutamine in the cytosol and chloroplasts. Although the enzymatic characteristics of the GS1 isozymes are well known, their physiological functions in ammonium assimilation and regulation in roots remain unclear. In this study we show 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 observed under conditions with or without ammonium supply. Loss of GLN1;2 caused significant suppression of plant growth and glutamine biosynthesis under ammonium-replete conditions. In contrast, loss of GLN1;3 caused slight defects in growth and Gln biosynthesis that were only visible based on a comparison of the gln1;2 single- and gln1;2:gln1;3 double-mutants. GLN1;2, being the most abundantly expressed GS1 isoform, markedly increased following ammonium supply and its promoter activity was localized at the cortex and epidermis, while GLN1;3 showed only low expression at the pericycle, 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 in Arabidopsis that allow adaptation to an ammonium-replete environment.

Key words: Ammonium, Arabidopsis, glutamine, GS1, nitrogen, root.

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 (Sasakawa and Yamamoto, 1978; Gazzarrini et al., 1999; Gu et al., 2013). However, given that excessive ammonium supply may inhibit plant growth...
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 (Tobin and Yamaya, 2001; Lea and Azevedo, 2007). Glutamine synthetase (GS or GLN) catalyzes a reaction that incorporates ammonium into glutamate and generates 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, whilst the other is used for transport, storage, or further metabolism (Tobin and Yamaya, 2001). GS is categorized into two groups: (1) the cytosol-localized GS1 group, and (2) the GS2 group localized mainly in the 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-CO₂ conditions; however, this growth defect is rescued under high CO₂ 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 isoforms assimilate non-photosynthetic ammonium (Tobin and Yamaya, 2001). In addition to primary uptake and photorespiration, ammonium can originate in several metabolic processes, including nitrate reduction, phenylpropanoid metabolism, degradation of transported amides, and protein catabolism (Schjoerring et al., 2002; Li et al., 2014). Four GS1 isoforms of Arabidopsis, encoded by GLN1;1, GLN1;2, GLN1;3, and GLN1;4, 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 isoform suggests that GS1 functions in non-photosynthetic ammonium assimilation in monocotyledonous crop plants, such as rice (Tabuchi et al., 2005; Funayama et al., 2013) and maize (Martin et al., 2006; Cañas et al., 2010). Phylogenetic analysis further suggests key differences between crop and Arabidopsis GS1 amino acid sequences (Thomsen et al., 2014), and the isoform-specific physiological functions of GS1 in Arabidopsis have been only partially documented or studied, with a focus on their roles in nitrogen remobilization in aerial organs based on their predominant expression being found in vascular tissues (Thomsen et al., 2014; Guan et al., 2015).

Three previous studies have reported on the physiological functions of GS1 isoforms in Arabidopsis using reverse-genetic approaches (Lothier et al., 2011; Guan et al., 2015, 2016). 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 the 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 under nitrate-sufficient conditions; however, no significant difference in nitrogen remobilization was found. When ammonium was supplied as the sole nitrogen source after pre-culture in nitrate-sufficient conditions, GLN1;2 insertion lines developed root hairs and had 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;1 and the double-insertion line for GLN1;1 and GLN1;2 showed a decrease in seed yield, whereas the single-insertion line for GLN1;1 showed a yield comparable to the wild-type. The GLN1;2 promoter-dependent green fluorescent protein (GFP) showed fluorescence localized in the vascular cells of roots, petals, and stamens (Guan et al., 2015). A more recent study showed that GLN1;2 is the main isoform 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). However, there remains a need for an efficient method that minimizes the nitrate used in the nutrient solution.

The enzymatic characteristics of recombinant GLN1;2 and GLN1;3 suggest that these two GS1 isoforms 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 remains 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 a reverse genetic approach using T-DNA insertion mutants and promoter-GFP lines reporting their differential physiological functions and spatio-temporal regulation. The finding of ammonium-responsive regulatory sequences in the GLN1;2 gene promoter region further implies a distinct contribution of the GLN1;2 isoform to ammonium assimilation in roots under ammonium-replete conditions.

**Materials and methods**

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

Arabidopsis (Arabidopsis thaliana) accession line Columbia (Col-0) was used as the wild-type (WT). The following T-DNA insertion lines in the Col-0 genetic background were used: gln1;2-1 (At1g66230; 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 the 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 001 (5'-CCAGTACATTTAAACAGTCCGCAATGGTCT-3') and T-DNA RB 01 (5'-CGAATACAGTGATCCGTCGCGGCCCTGG-3') for the GLN1;2 gene, GLN1;2F (5'-ATGAGTCTCTTTGCGATCTGTGTT-3') and GLN1;2R (5'-GGTCTTCAAAGTGTTAAGACCAACCGA-3') for the GLN1;3 gene, GLN1;3F (5'-ATGTCCTGCTTCAGATCTCGTAA-3') and GLN1;3R (5'-TCAACGCGATGTGTCGCTCAAGGC-3').

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

**Phenotypic analysis**

Three to five Arabidopsis seeds were germinated on water-moistened rock wool for 4 d in the dark, and single seedlings were selected. Plants were transferred to a hydroponic nutrient solution described
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by Loqué et al. (2006) with modifications. The modified hydronomic solution was buffered with 5 mM 2-(N-morpholino)ethanesulfonic acid (MES) adjusted to a pH of 5.8 with KOH, and 2 mM NH₄NO₃ was removed to be replaced with 10 μM KNO₃ and various concentrations of NH₄Cl, given that a small amount of nitrate alleviates the detrimental effects of pure ammonium nutrition (Krouk et al., 2006; Garroca et al., 2010). The nutrient solution was always buffered with MES. Plants were grown in three sizes of pots in preculture 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 0.71 l of nutrient solution. Second, at ammonium concentrations of 0.1, 0.3, 0.5, and 1 mM, 220 plants (44 plants per line, five genotypes) were grown in a 2-l plastic container filled with 2 l of nutrient solution. Third, at ammonium concentrations of 0.1 or 3 mM, 120 plants (17 plants per line, seven genotypes) were grown in a 5.9-l plastic container filled with 5 l of nutrient solution. 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 d 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 0.71 l of hydronomic solution and covered with the resin plate. The hydronomic solution was exchanged twice weekly. Plants were grown in a climate chamber (Biotron LPH-350S, Nippon Medical and Chemical Instruments Co., Ltd. Tokyo, Japan; 10/14 h light/dark, 22 °C, 60% humidity, and 160 μmol m⁻² s⁻¹ total 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 CaSO₄ solution for 1 min before harvest.

Samples were collected in envelopes or 2-ml safe-lock tubes (Eppendorf Co., Ltd., Tokyo, Japan). The samples were dried in an oven at 80 °C for several days. A zirconia bead was added to the 2-ml safe-lock tubes for milling samples. 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; 10/14 h light/dark, 22 °C, 60% humidity, and 160 μmol m⁻² s⁻¹ total 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 CaSO₄ solution for 1 min before harvest.

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Samples were frozen in liquid nitrogen immediately after measurement of the fresh weight using a 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 d 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 from one experiment are shown in the figures.

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

The GLN1;2 upstream region was amplified from Col-0 genomic DNA by PCR. KOD -Plus- DNA polymerase (Toyobo Co., Ltd, Osaka, Japan) was used in the PCR with gene-specific primers, GLN1;2P5097L_F: (5'-GGGATCCGGATGATGATGATGATAAAGATATATAA3') and GLN1;2P2501I_L: (5'-CGGATCCATTTTTCGAAGACATCCACACTCA3'). The entire GLN1;2 promoter region at different lengths (5697 bp or 2501 bp) was then fused with enhanced green fluorescent protein (GFP, Takara Bio Inc.) using the NcoI restriction site designed in the promoter region at different lengths (5697 bp or 2501 bp) and the region upstream of the GLN1;2 start codon was tagged with restriction sites for BamHI. The partial fragment of the GLN1;2 promoter region at different lengths was then fused with the upper region of the 2501-bp GLN1;2 promoter using the BamHI restriction site designed in the GLN1;2P2_R_02 primer. The entire GLN1;2 promoter region at different lengths (5372, 3822, 3624, 3604, 3563, 3522, or 3430 bp) was then fused with enhanced GFP (Takara Bio Inc.). The GLN1;2 promoter-GFP fragment was ligated to a pBTH101 (Clontech, Palo Alto, CA) binary vector, as previously described by 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). The GLN1;3 promoter-GFP lines originated from our previous study (Ishiyama et al., 2004).

Plants were grown in hydronomic culture or on vertical agar plates. In the hydronomic culture, plants were grown for 6 weeks in nutrient solution containing 0.1 or 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 the objective lens. 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 the spectral detector of the Nikon C1si System. Plants that were cultured on vertical agar plates were placed in a growth cabinet at 22 °C with 60% relative humidity under 16/8 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 (Fujitani et al., 1992) containing 7 mM nitrate as the major nitrogen source for 14 d; (2) plants were transferred to nitrogen-free MGRL medium and pre-cultured for 3 d to facilitate nitrogen starvation; and (3) plants were then transferred again to N-free MGRL medium either supplemented with 10 mM ammonium as the sole nitrogen source or containing no nitrogen source and incubated for 24 h for confocal microscopy or 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 described previously (Ishiyama et al., 2004). Images were processed in Adobe Photoshop.

Quantitative real-time 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 grown hydronically in nutrient solution with 0.1, 1, or 3 mM NH₄Cl and 10 μM KNO₃, for 6 weeks. Total RNA was extracted with an RNAeye 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 was 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 LightCycler® 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, and 60 °C or 65 °C for 34 s. SYBR Premix Ex Taq™ II (Takara Bio Inc.), 2 μl complementary (c) cDNA sample as a template, and 0.4 μM of each gene-specific primer were reacted. Gene-specific primers for GLN1;1, GLN1;2, and GLN1;3 were selected on the basis of the melting curves and the size of the PCR products. The melting temperatures of GLN1;1-specific primers were 62 °C, and the size of the PCR product was 113 bp. The melting temperatures of GLN1;2-specific primers were 74 °C, and the size of the PCR product was 121 bp. The melting temperatures of GLN1;3-specific primers were 71 °C, and the size of the PCR product was 121 bp.
were GLN1;3-RTF (5'-TCCAACCAACAGAGGCACAAC-3') and GLN1;3-RTR (5'-ACCAGAATATACGCCCTCAACA-3'). GFP specific primers were 204F (5'-AGTGCTAGCGGTACCC-3') and 345R (5'-CCCTCGAACTCCACCTCGG-3'). Serial dilutions of plasmid were used as standards. Data were acquired and analyzed using 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. The fold-change in gene expression relative to that of the WT at 1 mM ammonium was determined on the basis of crossing-point (CP) values (Pfaffl, 2001). RT-PCR primers for GLN1;2-specific primers were Gln1;2RF and NK124 (5'-CGGATCATCCTTTC AAGGATACAGAGGAG-3'), for GLN1;3-specific primers they were NK145 (5'-ATGTCTCTGCTTTCAGATCTCGT A-3') and NK146 (5'-TCAACCGATATGCGTCTTCAGCG-3'), and UBQ2-specific primers were prepared following the method of Ishiyama et al. (2004).

Nitrogen and carbon content

Plants were grown in a nutrient solution containing either 0.1 or 1 mM NH4Cl as the major nitrogen source for 6 weeks. Plant samples were dried and powdered with a Tissue Lyser II (Qiagen, K.K.), 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 concentrations were determined using an elemental analyzer (Flash2000, Thermo Fisher Scientific K.K., Yokohama, Japan).

Measurement of free amino acids and ammonium

Plant samples were frozen in liquid nitrogen and then milled using the Tissue Lyser II at 23 Hz for 15 min. Samples were weighed with an ultra-microbalance (UMX2, Mettler Toledo International Inc., Tokyo, Japan) in tin capsules. The weights of samples were always between 1.000 and 1.050 mg. Nitrogen and carbon were determined using 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 the 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 from one experiment are shown.

Xylem sap preparation

Plants were grown hydroponically in nutrient solution (Loqué et al., 2006) for 42 d and transferred to a nutrient solution without nitrogen for 3 d. Plants were transferred again to the solution containing 0.1 or 3 mM NH4Cl and 10 μM KNO3 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 3 h into the light period. Experiments were repeated at least twice with similar results, and representative values from one experiment are shown.

Statistics

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

Results

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

We evaluated the growth of A. thaliana Col-0 (WT) in a nutrient solution containing 10 μM KNO3, and supplemented with 0.1, 0.3, 1, 2, 3, 5, or 10 mM NH4Cl. Figure 1A shows the phenotype of the WT plants after 6 weeks in a hydroponic culture. As can be seen, WT shoots showed maximal growth under 1–2 mM NH4Cl. However, growth was decreased when the concentration of NH4Cl in the nutrient solution was >2 mM. The shoot and root dry weights of the WT plants tended to decrease at 3 mM, and were reduced by half at 5 mM compared with those at 1 mM Fig. 1B. Growth was even more strongly inhibited at 10 mM.

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

Two T-DNA insertion lines for GLN1;2 (Fig. 2A) and those for GLN1;3 (Fig. 2B) were isolated from the WT. One of

Fig. 1. Growth of the 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 NH4Cl as the major nitrogen source, supplemented with 10 μM nitrate for 6 weeks. (B) Shoot and root dry weights of the same plants as in (A). Data are means ±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. (This figure is available in colour at JXB online.)
The T-DNA insertion line, SALK_102291, was identical to that of a previous study by Lothier et al. (2011), whilst SALK_148604 was identical to that of a study by Dragičević 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). However, 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 the T-DNA insertion lines for GLN1;2. GLN1;5 was not detectable in the roots (Fig. 2H).

Figure 3 shows the different contributions of GLN1;2 and GLN1;3 to ammonium nutrition. The GLN1;2 insertion lines showed a marked reduction in dry weight compared with the WT. In addition, supplying ammonium led to a dose-dependent reduction in dry weight of the GLN1;2 insertion lines, whereas the GLN1;3 insertion lines showed no reduction. The 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, B). Conversely, there were no significant differences between the WT and gln1;2 in the nutrient solution containing either 1 or 10 mM nitrate (Supplementary Fig. S2 at JXB online).

Figure 4 shows nitrogen and carbon concentrations in the shoots and roots of the GLN1 insertion lines. Carbon concentration in the shoots and roots ranged from 35 to 40%, and there were no significant differences with the WT (Fig. 4B). The total nitrogen concentrations in the WT ranged from 3 to 4% at 0.1 mM ammonium, and increased to 6–8% under 1 mM ammonium (Fig. 4A). Nitrogen concentrations in the GLN1;2 insertion lines were significantly higher than those in the WT at 0.1 mM ammonium, and were lower under 1 mM (Fig. 4A). The GLN1;3 insertion lines showed no changes in nitrogen concentration under either 0.1 or 1 mM ammonium in comparison with the WT (Fig. 4A).

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

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 the double-insertion line expressed neither GLN1;2 nor GLN1;3 (Fig. 6B). Statistical analysis of fresh weight is presented in Fig. 7. In the single-insertion gln1;2, the fresh weight was decreased by half under 3 mM ammonium conditions, whereas in the
single-insertion *gln1;3*, it was not much different from the WT. The fresh weight of *gln1;2:gln1;3* was significantly different from the single-insertion lines. Under 0.1 mM ammonium conditions, it was decreased by 36% compared with *gln1;2*, and decreased by 46% compared with *gln1;3*. Under 3 mM ammonium conditions, it showed 48% and 77% reductions, respectively (Fig. 7).

To distinguish the functions of two root GS1 isozymes in ammonium assimilation in Arabidopsis, the free amino acid and ammonium concentrations were compared between the WT and the *GLN1* insertion lines 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 the *GLN1;2* insertion lines (Fig. 8C), whereas glutamine (Fig. 8B) as well as total amino acid (Fig. 8A) concentrations were decreased. Supplementary Fig. S3 shows the amino acid composition in shoots and roots of the WT and insertion lines. Glutamine accounted for >40% of the total amino acids in the shoots and >70% in the roots at 3 mM ammonium. A loss of *GLN1;2* led to a decrease in the
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Fig. 5. Nutrient use efficiency in roots and shoots of the wild-type (WT) and the GLN1;2 and GLN1;3 insertion lines. (A) Usage index (UI) was calculated as $UI = Sw \times (Sw/N)$, where $Sw$ is shoot weight, and $N$ is nitrogen in organs. (B) Uptake efficiency (UpE) was calculated as $UpE = Nt/Ns$ where $Nt$ is total nitrogen in the plant and $Ns$ is nitrogen supply (g per plant). Data are means ± SD ($n = 6$) for the WT, and the GLN1;2 and GLN1;3 insertion lines (as indicated in the key). Plants were grown hydroponically for 6 weeks, and supplemented with either 0.1 or 1.0 mM ammonium, and 10 μM nitrate. One-way ANOVA followed by Bonferroni tests were used, and significant differences at $P < 0.05$ within each group are indicated by different letters.

Fig. 6. Isolation of the double-insertion line for GLN1;2 and GLN1;3. (A) Reverse transcription polymerase chain reaction (RT-PCR) analysis of root RNA from the 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 by 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, and supplemented with 0.1 mM ammonium and 10 μM nitrate as the major nitrogen source.

Fig. 7. Growth of the wild-type (WT) and the 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 the insertion lines for GLN1;2 and GLN1;3. (B) Shoot and root dry weights of the WT the GLN1;2 insertion line, the GLN1;3 insertion line, and the GLN1;2:GLN1;3 double-insertion line (as indicated in the key). Plants were grown for 6 weeks in nutrient solutions containing 0.1 or 3 mM ammonium and 10 μM nitrate as the nitrogen source. Data are means ± SD ($n = 6$). One-way ANOVA followed by Bonferroni tests were used, and significant differences at $P < 0.05$ within each group are indicated by different letters. (This figure is available in colour at JXB online.)

The ratio of glutamine but an increase in the of ratio serine in the whole plant at 3 mM ammonium. The ratios of aspartate, threonine, and alanine were increased in the 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 in these compounds in xylem exudates from the Arabidopsis GLN1;2 and GLN1;3 insertion lines after supplying ammonium. Figure 9 illustrates the changes in glutamine and ammonium concentrations in the xylem sap over 24 h after supplying ammonium. GLN1;2 insertion lines resulted in a 50% decrease in glutamine in comparison to the WT (Fig. 9A). The ammonium concentration was higher in GLN1;2 than in the WT (Fig. 9B).

**Fig. 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 T-DNA insertion lines for GLN1;2 and GLN1;3. (B) The concentration of ammonium in xylem sap of the WT, the GLN1;2 and GLN1;3 insertion lines, and the GLN1;2:GLN1;3 double-insertion line (as indicated in the key). Plants were grown for 42 d in nutrient solution containing 2 mM ammonium nitrate and then transferred to nutrient solution without nitrogen. After 3 d, the plants were transferred again 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. Data are means ±SD (n = 4). One-way ANOVA followed by Dunnett tests were used, and significant differences at P<0.05 between the WT and the GLN1;2 or GLN1;3 insertion lines are indicated with asterisks (*), and significant differences between the GLN1;2 insertion line and the GLN1;2:GLN1;3 double-insertion line are indicated with a circumflex (^).

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

The GLN1;3 insertion lines did not show reductions in dry weight under any conditions tested except for 0.5 mM ammonium supply (Fig. 3A, B), when root dry weight was decreased by 20-30% (Fig. 3B). Since variability was observed among
Glutamine synthetase isozymes in Arabidopsis roots.

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 differences between the WT and the GLN1;3 insertion lines were observed (Figs 3–5, 7, 9, and Supplementary Fig. S2), gln1;2 and the gln1;2:gln1;3 double-insertion line (Fig. 6) were compared under 0.1 and 3 mM ammonium. The GLN1;3 insertion lines showed no significant decrease in fresh weight under the tested conditions (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.

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). The GLN1;3 insertion lines showed no clear changes in ammonium concentration (Fig. 8C). No significant differences were observed in the concentrations of total amino acids (Fig. 8A) and glutamine (Fig. 8B) between gln1;3 and the WT. The total amino acids and glutamine in gln1;2:gln1;3 were lower than those in the gln1;2 shoots (Fig. 8A, B), whereas ammonium in the double-insertion line was higher than in gln1;2 (Fig. 8C). Supplementary Fig. 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 the conditions tested, the GLN1;3 insertion lines showed no statistical differences from the WT (Fig. 9).

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

Figure 10A summarizes the expression of GLN genes in Arabidopsis roots under 0.1, 1, and 3 mM ammonium supply.

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![Figure 10](image_url)

**Fig. 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 the 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, 1, or 3 mM ammonium (as indicated in the key) and 10 μM nitrate for 6 weeks. Ubiquitin2 (UBQ2) was used to standardize the signal intensity. Data are means ±SD (n=3 or 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. (B–M) Localization of the promoter activities of GLN1;2 (B, D, F, H, J, L) and GLN1;3 (C, E, G, I, K, M). Transgenic plants expressing either the GLN1;2 promoter:GFP or the GLN1;3 promoter:GFP fusion gene constructs were grown for 6 weeks in nutrient solutions containing 0.1 mM (B, C, F, G, J, K) or 3 mM (D, E, H, I, L, M) ammonium and 10 μM nitrate as the nitrogen source. Whole-mount images from root tips (J–M), root hair zones (F–I), and mature parts (B–E) were acquired by confocal laser scanning microscopy. Scale bars represent 50 μm.
Roots greatly 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 conditions (Fig. 10A). GLN1;2 accounted for only 34% of the total GLN transcripts at 0.1 mM ammonium supply, but for almost 80% at 1 and 3 mM. GLN1;5 was not detectable.

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

To identify the regulatory region for the 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 Supplementary Fig. S5). The full-length promoter, containing a genomic region 5697 bp upstream of the GLN1;2 translational start codon, responded to ammonium in the growth medium and this resulted in 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 in GFP levels up to three-fold those under the control nitrogen-starved conditions (Fig. 11A and Supplementary Fig. S5). The induction of GFP accumulation, driven by this full-length promoter, was consistent with the increased accumulation of GLN1;2. Following the 5′-deletion series of the GLN1;2 promoter-GFP constructs, there was no great difference in the fold-change induction of GFP expression as far as the position −3604. However, truncation of the promoter to −3563 drastically reduced the GFP expression (Fig. 11A). Nevertheless, the endogenous GLN1;2 responded to the ammonium supply.

Discussion

Earlier studies showed that a small amount of supplied nitrate (Krouk et al., 2006; Yuan et al., 2007; Garnica et al., 2010) 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 was evident that the optimal ammonium concentration in the nutrient solution was 1 or 2 mM. The phenotypes observed below 3 mM ammonium were consistent with general ammonium assimilation but not with ammonium toxicity (see Supplementary Fig. S1).

Three independent T-DNA insertion lines for GLN1;3 and GLN1;2 (Fig. 2) and a double-insertion line for GLN1;2 and GLN1;3 were isolated (Fig. 6). The growth of the 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 and De Smet, 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 the xylem was partly dependent on GLN1;3. The growth and localization might suggest that the GLN1;3 assimilates concentrated symplastic ammonium around the stele. These findings extend the function of GLN1;3 from enzymatic characteristics to physiological functions within the plant. A previous study 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 differences in spatial and temporal expression of GLN1;2 and GLN1;3 determine 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 the root vascular tissues (Ishiyama et al., 2004; Lothier et al., 2011; Guan et al., 2015). In the present study, the GLN1;2 promoter was longer than that in previous studies because the shorter promoter (Ishiyama et al., 2004) did not respond to the ammonium supply (Fig. 10). The longer GLN1;2 promoter-GFP showed the localization of GLN1;2 in the epidermis and cortex under ammonium supply (Figs 10, 11, and Supplementary Fig. S4). The promoter deletion analysis suggested that at least the sequences between −3604 and −3563 bp are necessary to enhance GLN1;2 transcriptional activity in response to ammonium supply in the roots. 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), namely DNA-binding with one finger (DOF) (Yanagisawa, 1996), WRKY, bHLH, and viral core enhancer. This is in good agreement with previous studies suggesting DOF-dependent nitrogen metabolism (Yanagisawa et al., 2004) and DOF-dependent GLN expression (Rueda-López et al., 2008). GLN1;2 accumulation in response to ammonium supply initially occurs in the epidermis cell layers of Arabidopsis roots, where the 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 (Lothier et al., 2011; Guan et al., 2016). 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 higher concentrations of 2–20 mM, as shown in previous studies (Lothier et al., 2011; Guan et al., 2016) but also at lower concentrations of 0.3 mM (Fig. 3). Because ammonium in the soil solution varies from 0.1 to 0.8 mM (Miller et al., 2007), the presence of such a 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 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 roots (Figs 2 and 10), the five GLN genes may reflect the population of root GLN. Increasing the ammonium concentration severely inhibited 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 those of previous studies (Lothier et al., 2011). In addition to those phenotypes, GLN1;2 insertion dramatically increased the free ammonium concentration not only in the plant organs but also in xylem exudate, whereas free glutamine concentration was decreased (Figs 8, 9, and Supplementary Fig. S3). Xylem sap analysis indicated that GLN1;2-dependent ammonium assimilation mainly occurred in roots when the 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 accounted for approximately 15% in the shoots at 3 mM ammonium (see Supplementary Fig. S3), whereas arginine accounted for only <1% in nitrate-grown plants (Lothier et al., 2011). Due to its having 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 the shoots.

Neither the GLN1;2 nor the 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), which showed a reduction in biomass in rosette leaves of the GLN1;2 insertion line when plants were grown in 10 mM nitrate as the sole nitrogen supply, whereas there was no difference under the 2 mM nitrate supply. The reason for the mismatches between the two studies could be explained by the different culture conditions and genetic backgrounds used. There were differences in temperature, light, and nutrient (besides nitrate) concentrations. A previous study used the GLN1;2 insertion line in Arabidopsis Ws as the genetic background, whereas the present work used Col. The growth of Ws and Col show differences under nitrate-supply conditions (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 is not assimilated by GLN1;2. Although the present study provides insights into the physiological functions of GLN1;2 and GLN1;3, they are not the only GLN1 isozymes expressed in the roots of Arabidopsis. It will be necessary to investigate the functions of GLN1;1 and GLN1;4 with high-affinity ammonium to elucidate the full set of ammonium-assimilatory mechanisms in Arabidopsis plants.

Supplementary data

Supplementary data are available at JXB online.

Fig. S1. Growth of the WT under various concentrations of either nitrate or ammonium.

Fig. S2. Growth of the WT and T-DNA insertion lines for GLN1;2 and GLN1;3 under 1 or 10 mM nitrate supply.

Fig. S3. Amino acid composition in the WT and T-DNA insertion lines for GLN1;2 and GLN1;3 under 0.1 or 3 mM ammonium supply.

Fig. S4. Localization of the promoter activities of GLN1;2 and GLN1;3 on a vertical agar culture.

Fig. S5. The expression of GFP and GLN1;2 in GLN1;2 promoter lines in roots.
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