Cytosolic glutamine synthetase isozymes play redundant roles in ammonium assimilation under low-ammonium conditions in roots of *Arabidopsis thaliana*

Noriyuki Konishi$^{1,2}$, Masahide Saito$^1$, Fumi Imagawa$^1$, Keiich Kanno$^1$, Tomoyuki Yamaya$^1$, and Soichi Kojima$^1$*

$^1$Graduate School of Agricultural Science, Tohoku University, 468-1 Aoba, Aramaki, Sendai 980-0845, Japan
$^2$Division for Interdisciplinary Advanced Research and Education, Tohoku University, 6-3 Aoba, Aramaki, Sendai 980-8578, Japan

Ammonium is a major nitrogen source for plants; it is assimilated into glutamine via a reaction catalyzed by glutamine synthetase (GLN). *Arabidopsis* expresses four cytosolic GLN genes, *GLN1;1*, *GLN1;2*, *GLN1;3*, and *GLN1;4*, in roots. However, the function and organization of these GLN1 isozymes in ammonium assimilation in roots remain unclear. In this study, we aimed to characterize the four GLN1 isozymes. The levels of growth of wild type and *gln1* single- and multiple-knockout lines were compared in a hydroponic culture at ammonium concentrations of 0.1 and 3 mM. Under the low-ammonium concentration, in single mutants for each *GLN1* gene, there was little effect on growth, whereas the triple mutant for *GLN1;1*, *GLN1;2*, and *GLN1;3* grew slowly and accumulated ammonium. Under the high-ammonium concentration, the single mutant for *GLN1;2* showed 50% decreases in fresh weight and glutamine, whereas the other *gln1* single mutants did not show notable changes in the phenotype. The double mutant for *GLN1;1* and *GLN1;2* showed less growth and a lower glutamine concentration than the single mutant for *GLN1;2*. Promoter analysis indicated an overlapping expression of *GLN1;1* with *GLN1;2* in the surface layers of the roots. We thus concluded that: 1) at a low concentration, ammonium was assimilated by *GLN1;1*, *GLN1;2*, and *GLN1;3*, and they were redundant; 2) low-affinity *GLN1;2* could contribute to ammonium assimilation at concentrations ranging from 0.1 to 3 mM; and 3) *GLN1;1* supported *GLN1;2* within the outer cell layers of the root.

Keywords
Arabidopsis, ammonium, metabolism, root, glutamine synthetase

Correspondence
Dr. Soichi Kojima
E-mail, soichi.kojima.a2@tohoku.ac.jp

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Introduction
The analysis of ammonium assimilation in roots at various concentrations is vital for three important reasons: 1) ammonium and nitrate are the predominant nitrogen sources for plants in most soils (von Wirén et al. 2000); 2) ammonium inhibits plant growth at high concentration (Britto and Kronzucker 2002, Hachiya et al. 2012, Li et al. 2014); and 3) soil ammonium concentration fluctuates due to nitrogen fertilization, nitrification, and ammonia emission (Riley et al. 2001, Martins et al. 2015, dos Santos et al. 2016) in agricultural fields.

Most ammonium is assimilated in the roots and then translocated to the shoots through the xylem, mainly in amide forms such as glutamine and asparagine (Kiyomiyaya et al. 2001, Andrews et al. 2013, Yamaya and Kusano, 2014). Ammonium is first incorporated into glutamine in an ATP-dependent reaction catalyzed by glutamine synthetase (GS or GLN) (Tobin and Yamaya 2001, Lea and Azevedo 2007, Chardon et al. 2012, Thomsen et al. 2014). Cytosolic GLN1 is the major form in roots, while both GLN1 and plastidic GLN2 are expressed in shoots (Sakakibara et al. 1996, Ishiyama et al. 2004a and 2004b, Orsel et al. 2014). There are 3–16 GLN1 genes in the genome of higher plants (Orsel et al. 2014), and several GLN1 genes are expressed in roots (Sakakibara et al. 1996, Ishiyama et al. 2004a and 2004b, Tabuchi et al. 2007, Goodall et al. 2013, Orsel et al. 2014).

A major current focus in studies on ammonium assimilation is to understand the physiological functions of individual GLN1 isozymes. The Arabidopsis genome encodes five GLN1 genes (Thomsen et al. 2014). In previous reports, the importance of GLN1;2 in shoots was indicated (Lothier et al. 2011; Guan et al. 2016; Guan and Schjørring 2016). For example, in a GLN1;2 knockout line, there was a decrease in rosette fresh weight upon supplying 10 mM nitrate, although there was no obvious growth difference upon supplying 2 mM nitrate (Lothier et al. 2011). The expression of GLN1;2 was also increased by the supply of 20 mM ammonium in shoots, and gln1;2 mutants grew slowly upon the supply of 2–20 mM ammonium (Guan et al. 2016).

Arabidopsis expresses four GLN1 (GLN1;1, GLN1;2, GLN1;3, and GLN1;4) genes in roots (Ishiyama et al. 2004b). A study revealed that the recombinant GLN1 isozymes exhibit different enzymatic characteristics (Ishiyama et al. 2004b); specifically, GLN1;1 and GLN1;4 exhibit high-affinity for ammonium while GLN1;2 and GLN1;3 exhibit low affinity for it (Ishiyama et al. 2004b). Our recent report focused on the roots; it described the contribution of two low-affinity GLN1 isozymes (GLN1;2 and GLN1;3) to ammonium assimilation in roots (Konishi et al. 2017). A hydroponic culture containing ammonium as a major nitrogen source revealed that gln1;2 grew more slowly and accumulated more ammonium in the hydroponic nutrient solution containing ammonium at a concentration above 1 mM, whereas gln1;3 did not exhibit obvious changes (Konishi et al. 2017). The double knockout of GLN1;2 and GLN1;3 led to decreases in fresh weight and also glutamine concentration in the xylem sap (Konishi et al. 2017). Promoter activity of GLN1;2 was localized in epidermis and cortex in the root, whereas that of GLN1;3 was localized in the pericycle upon the supply of a high level of ammonium (Konishi et al. 2017). These results suggest the essential contribution of GLN1;2 to ammonium assimilation in the surface cell layers of roots and the non-essential contribution of GLN1;3 in the root pericycle (Konishi et al. 2017).

Nevertheless, the GLN1 isozyme responsible for the assimilation of lower concentrations of ammonium has not been identified because gln1;2 and gln1;3 single mutants do not show any notable phenotypes under lower-ammonium conditions (Konishi et al. 2017). Since the expression levels of GLN1;1 and GLN1;4 are high in low-nitrogen conditions (Lothier et al. 2011,
Konishi et al. 2017), high-affinity GLN1 isozymes (GLN1;1 and GLN1;4) are expected to be the major isozymes for the utilization of low concentrations of ammonium in roots.

To date, little information has been reported on the function of GLN1;4, while the functions of GLN1;1 have been reported by using reverse genetic approaches; however, the functions of GLN1;1 are still unknown under limited ammonium conditions (Guan et al. 2015, Guan et al. 2016).

The purpose of this study is to describe and examine the contribution and organization of GLN1 isozymes in ammonium assimilation in roots under a low ammonium concentration. Specifically, the present study analyzes the functions of four cytosolic GLN1 isozymes (GLN1;1, GLN1;2, GLN1;3, and GLN1;4) under low and high ammonium concentrations in Arabidopsis roots by using gln1 single-, double-, and triple-knockout lines and promoter-GFP lines. Comparison between gln1 single mutants and gln1 multiple mutants revealed the following findings: 1) at low ammonium concentrations, GLN1;1 and GLN1;4 have overlapping functions; 2) the high-affinity GLN1;1 can be compensated for by the low-affinity GLN1;2 and GLN1;3; 3) at high concentrations of ammonium, GLN1;2, with support from GLN1;1, is responsible for assimilating ammonium within the outer cell layers of the roots.

**Results**

**Knockout of high-affinity GLN1 did not change plant growth in ammonium nutrient supply**

To investigate the contribution of high-affinity GLN1 isozymes, GLN1;1 and GLN1;4, single mutants for GLN1;1 (gln1;1-4, Fig. 1a) and GLN1;4 (gln1;4-1 and gln1;4-2, Fig. 1b), and a double mutant for GLN1;1 and GLN1;4 (gln1;1:gln1;4) were prepared. RT-PCR analysis indicated a loss of GLN1;4 mRNA in gln1;4 and gln1;1:gln1;4, and a loss of GLN1;1 mRNA in gln1;1 and gln1;1:gln1;4 (Fig. 1c). Single and double gln1;1 and gln1;4 knockout lines were grown in a hydroponic solution containing either 0.1 or 3 mM ammonium as a major nitrogen source (Fig. 1d). Neither gln1;1 nor gln1;4 showed a significant difference in the biomass under the lower-ammonium condition (Fig. 1e). The fresh weight of the roots of gln1;1-4 and gln1;1:gln1;4 was slightly decreased under high ammonium supply, whereas other mutants, including the double mutant for GLN1;3 and GLN1;4 (gln1;3:gln1;4), did not differ from the wild type in this regard (Fig. 1e). In the low-ammonium condition, there were no significant differences in total amino acids (Fig. 2a) and ammonium (Fig. 2c) between the wild type and mutants, whereas glutamine (Fig. 2b) slightly increased in all mutants compared with the level in the wild type. In the high-ammonium condition, there were no significant differences in total amino acids (Fig. 2a), glutamine (Fig. 2b), and ammonium (Fig. 2c) between the wild type and mutants. These results indicate that the contribution of GLN1;1 or GLN1;4 to ammonium utilization at a lower-ammonium concentration can be fully compensated by the other types of GLN1.

**Generation of a triple-knockout line**

The growth of GLN1;1 and GLN1;4 mutants under nitrogen-limited conditions suggests some functional redundancy of GLN1 genes in ammonium assimilation. Furthermore, the expression patterns of GLN1 genes that we previously determined (Konishi et al. 2017) support the non-negligible contribution of GLN1;2 under ammonium-limited conditions. To address the issue of functional redundancy, we constructed double mutants, gln1;1:gln1;2, gln1;1:gln1;3, and gln1;3:gln1;4, in this work, and gln1;2:gln1;3 (Konishi et al. 2017), and a triple knockout line (gln1;1:gln1;2:gln1;3, tko) created by genetic crossing. The RT-PCR analysis confirmed that none of the three GLN1s were detectable in the tko (Fig. 3a).
We attempted to cross gln1;2 with gln1;4, but this was not successful. Double knockout of GLN1;2 and GLN1;4 seemed to have a lethal effect on plants. To isolate gln1;2:gln1;4 double mutants, gln1;2-1 and gln1;4-1 were crossed and self-fertilized, and the segregation of the knockout line was determined in the F2 generation. Genotypes of GLN1;2 and GLN1;4 in the F2 generation were analyzed by PCR using gene-specific primers for GLN1;2 and GLN1;4. PCR analysis showed that there were no gln1;2:gln1;4 homozygous plants among 182 plants from the F2

Figure 1. The gln1;1 gln1;4 double knockout line does not show growth inhibition under the low-ammonium condition

The position of T-DNA insertion in gln1;1-4 (a), and in gln1;4-1 and gln1;4-2 (b). Exons are illustrated as filled boxes, lines represent introns, and open boxes correspond to 5'- and 3'-untranslated sequences. Arrows indicate the positions of gene-specific primers used for RT-PCR. RT-PCR analysis of root RNA from wild type (Col-0), gln1;1, gln1;4, and gln1;1:gln1;4 (c). Plants were grown in a hydroponic solution containing 0.1 mM ammonium for 6 weeks. Images of the gln1;1 and gln1;4 knockout lines and their corresponding wild type (Col-0), as well as of the double mutant, gln1;1:gln1;4, after growth in the hydroponic solution containing either 0.1 or 3 mM ammonium for 6 weeks (d). Fresh weights of shoots and roots of the wild type (Col-0) (black columns), gln1;1 (orange-red columns) and gln1;4 mutant lines (deep magenta columns), and gln1;1:gln1;4 (bubble gum column) and gln1;3:gln1;4 double knockout lines (light green column), with the same plants as in d (e). Bars indicate mean ± SD (n = 6). One-way ANOVA, followed by Bonferroni test, was performed, and significant differences at p < 0.05 within each group are indicated by different letters.
generation (Fig. S1a). We also attempted to isolate the gln1;1:gln1;3:gln1;4 triple mutant, but this was also unsuccessful. During the isolation, a T-DNA insertion line named line 98’ was isolated. PCR analysis indicated that T-DNA insertion in the genomic DNA of line 98’ was homozygous in GLN1;1 and GLN1;3, and heterozygous in GLN1;2 and GLN1;4. Line 98’ expressed neither GLN1;1 nor GLN1;3 but expressed GLN1;2 and GLN1;4 (Fig. S1b). A progeny test showed that T-DNA insertion in GLN1;4 was not found in the 183 plants from the self-propagation of line 98’ (Fig. S1c).

Triple knockout of GLN1;1, GLN1;2, and GLN1;3, but not the GLN1;1 single mutant, was associated with decreased biomass under low and high ammonium supply

The lines with gln1 knockout were grown in a hydroponic nutrient solution containing either low (0.1 mM) or high (3 mM) ammonium as the primary nitrogen source (Fig. 3b). The fresh weight of these mutants was compared with that of the wild type (Fig. 3c). Under the lower-ammonium condition, single mutants did not show a marked reduction in shoot biomass; only root biomass of the gln1;2 single mutants showed a 15% reduction. By contrast, double and triple mutants grew more slowly than the wild type (Fig. 3c). Neither gln1;1 nor gln1;3 showed a significant difference in biomass compared with the wild type; only gln1;2 showed a slight (15%) but significant decrease in root fresh weight (Fig. 3c). The fresh weights of gln1;1:gln1;2 and gln1;2:gln1;3 decreased by 50% and 30%, respectively, while that of gln1;1:gln1;3 showed a slight decline in the root (Fig. 3c). The growth of tko was most severely impaired. Specifically, the loss of GLN1;1, GLN1;2, and GLN1;3 led to a 75% decrease of biomass upon the supply of 0.1 mM ammonium (Fig. 3c). The growth phenotypes of gln1;1:gln1;2, gln1;1:gln1;3, and gln1;2:gln1;3 could be considered to be synergistic (Fig. S2). This suggests that GLN1;1, GLN1;2, and GLN1;3 play functionally redundant roles in ammonium assimilation under the low-ammonium condition.

Under the higher-ammonium condition, GLN1;2 showed a unique contribution to ammonium assimilation. While the fresh weight of the GLN1;2 mutant decreased by half, neither gln1;1 nor gln1;3 showed notable changes in biomass (Fig. 3c). However, double and triple mutants revealed the contribution of GLN1;1 in the gln1;2 background. The fresh weight of gln1;1:gln1;2 was lower than that of gln1;2, and the fresh weight of tko was lower than that of gln1;2:gln1;3 (Fig. 3c). The differences of fresh weight between gln1;1:gln1;2 and gln1;2 and between tko and gln1;2:gln1;3 were approximately 25%. Double and triple knockout revealed the contribution of GLN1;3 only in the gln1;1 background but not in the gln1;2 background, suggesting an epistatic relationship between GLN1;2 and GLN1;3.

The RT-PCR analysis only showed the expression of GLN genes under the supply of 0.1 mM ammonium (Fig. 3a). Since this experiment was conducted with RNA from roots of plants grown with only a low concentration of ammonium, we tested the expression of GLN genes...
by quantitative RT-PCR under both 0.1 and 3 mM ammonium conditions again and performed a protein gel blot analysis upon the supply of 3 mM ammonium. Figure S3 illustrates the expression of GLN1 mRNA and the accumulation of GLN1 protein in roots. Consistent with Fig. 3, no expression of GLN1;1,
Figure 4. Ammonium-dependent nitrogen and carbon concentrations in roots and shoots of wild type and gln1 mutants

Nitrogen (a) and carbon (b) concentrations in shoots and roots of the wild type (Col-0), GLNI single mutants (gln1;1, gln1;2, and gln1;3), double mutants (gln1;1:gln1;2, gln1;1:gln1;3, and gln1;2:gln1;3), and triple mutant (gln1;1:gln1;2:gln1;3). Plants were grown in hydroponic culture with 0.1 or 3 mM ammonium and 10 µM nitrate for 6 weeks. Data are presented as mean ± SD (n = 6). One-way ANOVA, followed by Bonferroni test, was performed, and significant differences at p < 0.05 within each group are indicated by different letters.
GLN1;2, or GLN1;3 was detected in the roots under both ammonium conditions (Fig. S3). Knockout of GLN1;2 led to a decrease of GLN1;1, GLN1;4, and GLN2 (Fig. S3) under a low ammonium supply, suggesting the dependence of these GLN genes on GLN1;2. Interestingly, the GLN1;4 transcript level was highest in the tko (Fig. S3) at higher ammonium supply, suggesting the possible compensatory upregulation of GLN1;4 in the absence of other GLN1 genes.

Soluble proteins from roots of hydroponically grown Arabidopsis were subjected to a protein gel blot analysis using an antibody raised against rice GS1 (Ishiyama et al. 2004b) to evaluate the amount of GLN1 protein. A specific band at 40 kDa (GS1) was detected (Fig. S4); however, GS2 (44 kDa) was hardly detectable in roots. Since antibody staining showed the changes in the level of GLN1 protein in knockout mutants, the band intensity was quantified using the image processing program, ImageJ. While only gln1;1:gln1;2 and tko showed significant decreases in GLN1 protein at 0.1 mM ammonium, in all mutants with the gln1;2 genetic background, there were always significant decreases in GLN1 protein at 3 mM ammonium, indicating that GLN1;2 was the major form of GLN1 protein under the higher-ammonium condition.

Nitrogen and carbon concentrations were determined in the single and multiple mutants (Fig. 4) under conditions with the supply of 0.1 or 3 mM ammonium and 10 µM nitrate for 6 weeks. Data are presented as mean ± SD (n = 3). One-way ANOVA, followed by Bonferroni test, was performed, and significant differences at p < 0.05 within each group are indicated by different letters.

To investigate the contribution of individual GLN1s to ammonium assimilation, the total amino acid, glutamine, and ammonium concentrations were determined in plants (Fig. 5). Amino acid composition is summarized in Figure S5, which depicts the impact of GLN1 on amino acid metabolism.

Furthermore, to estimate the capacity for ammonium assimilation in the roots, the concentrations of ammonium and glutamine were determined in xylem sap (Fig. S6). In the low-ammonium condition, total amino acid and glutamine levels of tko were significantly higher than those of the wild type (Fig. 5a and 5b). Double mutants, gln1;1:gln1;2 and gln1;1:gln1;3,
also accumulated glutamine (Fig. 5a and 5b).

This accumulation of glutamine in tko might reflect its ammonium accumulation. Indeed, the ammonium concentration of tko was considerably increased, whereas gln1 single and double mutants did not show any differences from the wild type (Fig. 5c). The ammonium concentration of tko was 60 times higher in roots and 13 times higher in shoots than that of the wild type (Fig. 5c). Among the single mutants, gln1;2 showed marked decreases in total amino acids and glutamine (Fig. 5a and 5b). There was also a significant difference in the amino acid composition between low (Fig. S5a and S5b) and high ammonium supply (Fig. S5c and S5d). Wild type plants had a high ratio of alanine to total amino acids under the condition with 0.1 mM ammonium. The share of alanine reached 48.3% in root and 44.5% in shoot. Aspartic acid (4.8% in root and 8.9% in shoot) and glutamic acid (13.8% and 20.8%) were also highly accumulated, and lysine (2.1% and 1.7%), valine (2.7% and 1.7%), and threonine (4.1% and 2.4%) levels were relatively high. Under the condition with 3 mM ammonium, glutamine was the most abundant amino acid. The
The share of glutamine reached 61.9% in root and 41.4% in shoot. Asparagine (6.0% and 12.6%) and serine (6.3% and 10.5%) were also accumulated. Notably, arginine was highly accumulated in shoot: it rose by 11.8% in shoot under the condition with 3 mM ammonium. Finally, ammonium supply did not change either proline or glycine accumulation.

The amino acid composition in tko showed a great difference. With the lower supply of ammonium, its level of glutamic acid dropped from 13.8% to 5.4% in root and 20.8% to 10.2% in shoot; moreover, aspartic acid dropped from 4.8% to 3.9% in root and 8.9% to 6.5% in shoot in tko. The proportions of lysine, valine, and threonine were all decreased in tko. Conversely, the proportions of serine and glutamine were increased. Serine increased from 4.7% to 7.4% in root.

### Supplemental Figure S1. Segregation of T-DNA insertion in GLN1;2 and GLN1;4

The segregation of T-DNA insertion in the F2 generation of the line obtained from crossing between *gln1;2* and *gln1;4* (a). RT-PCR analysis (b) and progeny test (c) of the self-propagation of line 98’. Genotypes of *GLN1;2* and *GLN1;4* were confirmed by PCR analysis with gene-specific primers. The symbol “+” indicates no T-DNA insertion, while “−” indicates T-DNA insertion in either *GLN1;2* or *GLN1;4*.

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**total 183 plants**
and 7.7% to 23.8% in shoot, while glutamine increased from 6.5% to 11.5% in root and 4.0% to 17.4% in shoot in tko. Under the high-ammonium condition, the proportions of glutamine and asparagine in tko were lower than those of the wild type. Glutamine decreased from 61.9% to 14.1% in root and 41.4% to 15.4% in shoot, while asparagine dropped from 6.0% to 3.4% in root and 12.6% to 4.4% in shoot in tko. Conversely, the proportion of alanine in tko was higher than that in the wild type. The proportion of arginine in shoot was also lower in tko.

Xylem sap analysis suggested that GLN1;1 and GLN1;2 contribute to the ability of roots to perform ammonium assimilation (Fig. S6) because the ammonium concentration of xylem sap was increased in gln1;1:gln1;2 and tko. The concentrations of ammonium in gln1;1:gln1;2 and tko were significantly higher than those of the wild type under all ammonium concentration conditions tested. Conversely, the concentration of total amino acid, especially glutamine, was decreased in gln1;2, gln1;1:gln1;2, and tko (Fig. S6b and S6c).

**The promoter activities of GLN1;1 were localized in surface cell layers, and GLN1;4 was strongly expressed in the pericycle of basal parts of lateral roots**

Figure 6 illustrates the localization of GLN1;1 and GLN1;4 promoter activities under low- and high-ammonium conditions. The promoter activities of GLN1;1 were localized in the epidermis, cortex, and root tip under low ammonium concentrations (Fig. 6a, e, and j). The GLN1;1 promoter-dependent GFP signal was also observed in the endodermis in the mature regions of the root (Fig. 6a). With increasing ammonium concentration, GLN1;1-dependent signals disappeared from the endodermis of mature regions and the cortex of elongating regions (Fig. 6c and 6h). The GLN1;4-dependent GFP signal was not detected at the root tip (Fig. 6k and 6m). These GLN1;4 promoter activities disappeared under the high ammonium supply (Fig. 6d and 6i).

**Discussion**

Glutamine synthetase genes are part of a multi-gene family in the genome of most plant species. Their products, GLN1 and GLN2, are categorized by their subcellular localization (Thomsen et al. 2014). Analysis of plant genome sequences has revealed five GLN1 homologs in Arabidopsis, three in rice, five in maize, seven in wheat, and 16 in Brassica napus (Orsel et al. 2014). Substantial research in recent years has focused on the job-sharing of GLN1 genes to understand the links between nitrogen-related nutrition and plant growth. We describe here a reverse-genetic approach and promoter analysis in roots to identify the organization and contribution of GLN1 isozymes to ammonium assimilation in Arabidopsis roots.

Among the six GLN genes in Arabidopsis, GLN1;1, GLN1;2, GLN1;3, and GLN1;4 were highly expressed in roots (Ishiyama et al. 2004b). Nitrogen deficiency led to the upregulation of GLN1;1, GLN1;3, and GLN1;4 in roots (Konishi et al. 2017, Figure S5). A previous biochemical study indicated that recombinant proteins of...
both GLN1;1 and GLN1;4 had high-affinity to ammonium (Ishiyama et al. 2004b), suggesting a contribution of these two GLN1 isozymes to efficient ammonium assimilation in roots under nitrogen-limited conditions. However, neither single mutants for GLN1;1 and GLN1;4 nor the double mutant for these two GLN1 isozymes showed a reduction in biomass under ammonium-limited conditions (Fig. 1), suggesting that the GLN1 genes have redundant functions in ammonium assimilation in roots. Indeed, none of the gln1 single mutants (gln1;1, gln1:2, gln1:3, and gln1:4) showed notable phenotypic changes under low ammonium concentrations (Figs. 3, S3, 4, and 6). These results indicate that, in a single mutant of GLN1, compensation by other GLN1 isozymes could occur; thus, low concentrations of ammonium are probably assimilated by the redundant functions of GLN1 isozymes in Arabidopsis roots. It was surprising that the high-affinity GLN1;1 could be compensated for by the low-affinity GLN1;2 and GLN1;3. The concentration of free ammonium in roots may account for this compensation. The ammonium concentration of all knockout lines ranged from 0.5 to 1 mM, except that of tko (Fig. 5c). Previous work suggested that the affinity constants (Km) of GLN1;2 and GLN1;3 for ammonium are 2.5 and 1.2 mM, respectively (Ishiyama et al. 2004b), suggesting a possible contribution of these two GLN1 isozymes to the ammonium assimilation in the lower-ammonium condition presented in this study. A hydroponic culture solution containing ammonium at a concentration below 0.1 mM might reduce the effects of GLN1;2 and GLN1;3 to reveal those of GLN1;1 and GLN1;4. However, it would not be possible to conduct such a culture because 1) plants would suffer from severe nitrogen starvation and 2) the nitrogen starvation would trigger the degradation of nitrogen compounds to maintain the ammonium concentration (Bittanszky et al. 2015). In both of these cases, it is likely that the functions of GLN1;1 and GLN1;4 would not be revealed. Therefore, the generation of multiple mutants is a powerful tool to investigate the function of specific GLN1 enzymes.

Amino acid analysis indicated that a loss of function in GLN1 led to an imbalance of amino acid composition. The wild type accumulated Ala under the condition with 0.1 mM ammonium (Fig. S5). Pyruvate is one of the sources for Ala and Val synthesis (Hirai et al. 2004), and it is also one of the sources for the TCA cycle. Ammonium assimilation depends on 2-oxoglutarate, one of the components of the TCA cycle. The increase of Ala under the condition with low ammonium might have reflected the lower demand for pyruvate from the TCA cycle. Similar to Ala, Val was accumulated under the condition with 0.1 mM ammonium. Moreover, the wild type accumulated Gln, Asn, Ser, and Arg (Fig. S5) in 3 mM ammonium. These changes in amino acid levels were similar to those described in a previous work (Lemaire et al. 2008). Another study reported that a higher ammonium supply increases Gln (Konishi et al. 2014 and Kojima et al. 2014). Under high-nitrogen conditions, the increase of asparagine follows the increase of glutamine (Lea et al. 2007). The arginine molecule contains four nitrogen atoms and acts as a nitrogen reservoir (Lemaire 2008).
Triple mutations in \textit{GLN1} led to decreases of Glu, Asp, Lys, Val, and Thr (Fig. S5) at 0.1 mM ammonium. Nitrogen starvation was also reported to increase the levels of these amino acids (Krapp et al. 2011). Therefore, it can be assumed that a decrease of Gln synthesis in the triple mutant triggers severe nitrogen starvation. Interestingly, the triple mutant accumulated Gln at 0.1 mM ammonium. The lack of Gln synthesis probably inhibits the other amino acids, including Glu, since the synthesis of other
amino acids is dependent on the GS/GOGAT cycle. Because of the inhibition of ammonium assimilation in tko, plants might suffer from ammonium toxicity upon a low ammonium supply. Indeed, tko accumulated much more ammonium than the wild type (Fig. 5).

At higher ammonium, the concentrations of Gln and Asn in the triple mutant were lower than those in the wild type. This again indicates that Asn synthesis requires Gln in plants (Ohashi et al. 2015).

Under the higher ammonium condition, the expression of GLN1;1, GLN1;3, and GLN1;4 decreased, while that of GLN1;2 was increased. Previous articles described the essential role of GLN1;2 at an ammonium supply above a concentration of 1 mM (Lothier et al. 2011, Guan et al. 2016, Konishi et al. 2017). Our previous work

Supplemental Figure S5. Amino acid composition in shoots and roots of knockout lines for GLN1 under low- and high-ammonium conditions

The concentrations of amino acids were measured in shoots (b and d) and roots (a and c) of wild type (Col-0), GLN1 single mutants (gln1;1, gln1;2, and gln1;3), double mutants (gln1;1:gln1;2, gln1;1:gln1;3, and gln1;2:gln1;3), and the triple mutant (gln1;1:gln1;2:gln1;3). Plants were grown in a hydroponic culture with 0.1 (a and b) or 3 mM ammonium (c and d) and 10 µM nitrate for 6 weeks. Data are presented as mean ± SD (n = 3). One-way ANOVA, followed by Bonferroni test, was performed, and significant differences at p < 0.05 within each group are indicated by different letters.
Supplemental Figure S6. Amino acid and ammonium concentrations in xylem sap harvested from knockout lines for GLN1

The concentrations of ammonium (a), glutamine (b), and total amino acids (c) were measured in xylem sap of wild type (Col-0), GLN1 single mutants (gln1;1 and gln1;2), double mutants (gln1;1:gln1;2), and triple mutant (gln1;1:gln1;2:gln1;3, tko). Plants were grown for 42 days in a nutrient solution containing 2 mM ammonium nitrate and transferred to a nutrient solution without nitrogen. After 3 days, the plants were again transferred to a nutrient solution containing 0.1, 1, or 3 mM ammonium and 10 µM nitrate. After 24 h, plants were excised, and xylem sap was collected. Bars indicate mean ± standard deviation (SD) (n = 4). One-way analysis of variance (ANOVA), followed by Bonferroni test, was used, and significant differences at p < 0.05 within each group are indicated by different letters.
also showed the contribution of GLN1;2 upon a higher ammonium supply in hydroponic culture (Konishi et al. 2017). The addition of nitrate to the hydroponic nutrient solution could alleviate the toxicity of ammonium at a higher concentration (Fig. S6). The knockout in GLN1;2 led to a decrease in biomass, while neither gln1;1 nor gln1;3 showed a difference (Fig. 3).

However, gln1;1:gln1;2 showed considerable decreases in biomass (Fig. 3), nitrogen concentration (Fig. 4), and glutamine (Fig. 5), which were lower than those in gln1;2. These phenotypes found in this study were consistent with those observed in previous work (Guan et al. 2016). For example, Guan et al. (2016) reported that GLN1;1 supports GLN1;2 for overcoming the toxic effects of excessive levels (20 mM) of ammonium in Arabidopsis shoot. The double mutant gln1;1:gln1;3 showed significant decreases in fresh weight (Fig. 3) and also in glutamine concentration (Fig. 5). These results indicate that GLN1;2 can compensate for the single mutant in either GLN1;1 or GLN1;3, but not for their double mutant, suggesting that the contributions of GLN1;1 and GLN1;3 to ammonium detoxification in Arabidopsis roots are not negligible, although GLN1;2 shows the largest contribution.

The contributions of GLN1;1, GLN1;2, and GLN1;3 to biomass as determined in single mutants were not the same as those determined in the comparison between single mutants and double mutants or those determined in the comparison between double mutants and the triple mutant (tko; Fig. S2).

Supplemental Figure S7. A small amount of nitrate alleviated the ammonium toxicity in hydroponic culture

Wild type plants were grown in a hydroponic solution containing various concentrations of ammonium (0.1, 0.3, 1, 3, and 5 mM) with or without 10 µM nitrate for 6 weeks. Fresh weights without (orange-red column) and with nitrate (blue column). Bars indicate mean ± SD (n = 3–6). Significant differences between with nitrate condition and without nitrate condition, identified by Student’s t-test, are marked by Student's t-test, are marked with asterisks: *p < 0.05 and ***p < 0.005; n.s. indicates not significant.
The contribution of GLN1;1 and GLN1;2 in single mutants amounted to 0%–20% of that of the wild type, but this increased to 40%–60% in the double mutant under lower-ammonium conditions. In the knockout lines with an insertion in GLN1;3, the contributions of GLN1;1 and GLN1;2 amounted to 10%–35% of that of gln1;3, which increased to 65%–80% in the tko, suggesting the synergistic interactions of GLN1;1 and GLN1;2. Double mutation in GLN1;1 and GLN1;2 increased the nitrogen concentration upon a low-ammonium supply, in both gln1;1:gln1;2 and tko (Fig. 4). This might be partially explained by the decrease of biomass or the potential upregulation of ammonium uptake in both mutants. Recently, it was suggested that there is a link between the response of GLN1;2 and ammonium uptake capacity (Yasuda et al. in press). Interestingly, not only GLN1;1–GLN1;2 but also GLN1;2–GLN1;3 and GLN1;1–GLN1;3 showed synergistic interactions under low-ammonium conditions, suggesting the functional redundancy of these GLN1 isozymes in roots upon a low supply of ammonium. Since there was no obvious upregulation of mRNA levels in the knockout lines (Fig. S3), the compensation of GLN1 at the transcriptional level could be ruled out to explain the growth of single mutants. Thus, post-translational regulation might be responsible for their synergistic effect.

GLN1;1, GLN1;2, and GLN1;3 have been shown to interact with each other to form homo- and heterodecamers (Dragicevic et al. 2014). Furthermore, GLN1 proteins have been found to be regulated by post-translational modifications (Li et al. 2006). Thus, future work should focus on the post-translational regulation of GLN1 proteins to understand the mechanism behind the synergistic interactions of GLN1.

Promoter analysis indicated that GLN1;2, with support from GLN1;1, is responsible for assimilating ammonium within the outer cell layers of the root (Fig. 6, Konishi et al. 2017). This overlapping expression of GLN1;1 and GLN1;2 suggests that root surface cell layers are crucial sites for ammonium assimilation. Arabidopsis root expresses ammonium transporter to transport ammonium from the environment into root cells (Yuan et al. 2007).

AMT1 isozymes, AMT1;1, AMT1;2, AMT1;3, and AMT1;5, contribute to 95% of the ammonium acquisition under nitrogen-deficient conditions at the surface cell layers in the roots (Yuan et al. 2007). The localization of AMT1 was reported to overlap well with that of GLN1;1 and GLN1;2 (Yuan et al. 2007), suggesting that GLN1;1 and GLN1;2 could assimilate ammonium after uptake through AMT1s in the root surface cell layers.

Promoter analysis also indicated that GLN1;3 and GLN1;4 could assimilate the ammonium concentrated in the pericycle (Fig. 6, Konishi et al. 2017). The ammonium accumulation in tko was higher than that in gln1;1:gln1;2 (Fig. 5c), which allows estimation of the function of GLN1;3. Previously, we showed that GLN1;3 contributes to growth and glutamine loading to xylem sap under low-ammonium conditions (Konishi et al. 2017). Consistently, this work shows the higher ammonium accumulation in tko than in gln1;1:gln1;2, suggesting the contribution of GLN1;3 to ammonium assimilation around the pericycle. Neither single nor double mutants of GLN1;4 showed notable differences in growth (Figs. 1 and 2); however, several lines of evidence indicated the physiological function of GLN1;4: 1) tko was not lethal but could grow and achieve 25% of the fresh weight of the wild type (Fig. 3); 2) the expression of GLN1;4 was increased in roots of tko (Fig. 5); and 3) GLN1;4 promoter activity was found in the pericycle in low-ammonium conditions (Fig. 6). These results demonstrate the potential contribution of GLN1;4 and GLN2 to ammonium assimilation around the pericycle under nitrogen-deficient conditions.

Interestingly, neither gln1;2:gln1;4 nor gln1;1:gln1;3:gln1;4 was isolated (Fig. S1). Although the germination of the selfed progeny of
Methods

Isolation of knockout lines for GLN1;1 and GLN1;4

The Arabidopsis thaliana accession line Columbia (Col-0) was used as the wild type. The following knockout lines in the Col-0 genetic background were also used: gln1;1-4 (AT5G37600; GABI_265C09), gln1;4-1 (AT5G16570; SALK_042546), gln1;4-2 (SALK_007138), gln1;2-1, and gln1;3-1 (Konishi et al. 2017). Knockout lines for GLN1;1 and GLN1;4 were obtained from the Arabidopsis Biological Resource Center, self-fertilized, and selected for T-DNA homozygous plants. The T-DNA insertions were determined by genomic PCR using primers for gln1;1-4, GLN1;1 cDNA F2 (5’- CATCAA CCT TAA CCT CTCAGA CT -3’), GLN1;1 cDNA R2 (5’- TCTGCAATC AGTGAA GTGACA AT -3’), and GABI T-DNA LB o8409 (5’- ATTTGAGG TACGAGAGAG TAGTCTT CCTCATC -3’); for gln1;4-1, gln1;4 042546 F2 (5’-GTTTCTAGTAGCATATAAGG G3 (5’-TTGATCAGCTGGGAGAAG TT-3’), and T-DNA LB-01 (Konishi et al. 2017); and for gln1;4-2, gln1;4 genome 117504 F (5’-GGATGGAACTGAGCACCACACGACGGAA GC-3’), gln1;4 genome 117504 R (5’-AAGCTGGCCATCTTTCA-3’), and T-DNA LB-01. The law of inheritance in both lines. The P values of the chi-square tests were 3.19 x 10^{-42} and 7.66 x 10^{-107}, respectively (Fig. S1). These results indicate the potential contribution of GLN1;4 in the reproductive stage. In future, it will be necessary to investigate the detailed functions of GLN1;4 for the productivity of Arabidopsis.

Isolation of multiple-knockout lines for GLN1 genes

gln1;1-4 and gln1;2-1 or gln1;3-1 and gln1;4-1 were crossed, and double mutants (gln1;1:gln1;2 and gln1;3:gln1;4) were isolated. Moreover, gln1;1:gln1;2 and gln1;3:gln1;4 were crossed, and the double- (gln1;1:gln1;3) and triple-knockout lines (gln1;1:gln1;2:gln1;3) and line 98’ [gln1;1:GLN1;2 (+/-); gln1;3:GLN1;4 (+/-)] were isolated. T-DNA insertion types were determined by genomic PCR by using primers for gln1;1-4 (as aforementioned), gln1;2-1; gln1;2-1-2 mutant genome F (5’-GGTTGAGG TGGTCTGATGGCATGAG-3’), gln1;2-1 mutant genome R (5’-ACTTCA CGACATACATGTTGG TTAGA TGGTCTTGATGGCATGAG-3’), and GABI T-DNA LB-01, gln1;3-1; gln1;3-1 mutant genome F3 (5’-CGGATGGATGCTTTCTCCT -3’), gln1;3-1 mutant genome R3 (5’-CAGCTTGAGACRGGT -3’), and T-DNA LB-01, and gln1;4-1 (as aforementioned). Multiple-knockout lines were selected from self-fertilized F2 generation plants. The isolation of gln1;2:gln1;3 was as described previously (Konishi et al., 2017). gln1;2-1 and gln1;4-1 were crossed, self-fertilized, and the T-DNA insertion types were determined by genomic PCR. The T-DNA insertion types were determined by genomic PCR in the F2 plants of line 98’. Plants were grown on agar plates containing half-strength Murashige and Skoog medium (Murashige and Skoog, 1962) for 3 to 4 weeks. Genomic DNA was prepared from leaf samples.

Hydroponic culture

Hydroponic culture and plant harvesting were performed in accordance with the method of Konishi et al. (2017). Plants were grown in nutrient solution with 0.1 or 3 mM NH4Cl and 10 µM KNO3 for 6 weeks. Plants (34 plants per line, 8 genotypes) were precultured in a plastic container filled with 8 L of nutrient solution. Plants were transferred to a black acrylic resin plate from the preculture containers at 25 days after sowing. Plants were grown in a climate chamber under the following environmental conditions (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. The size of the tanks after the preculture was 0.8 L, and six to eight plants were transferred from the preculture. The composition of the
nutrient solution was described by Loqué et al. (2006) with modifications; it 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. We added a small amount of nitrate to alleviate the detrimental effects of pure ammonium nutrition.

**Quantitative real-time (q) PCR analysis and reverse transcription (RT)-PCR analysis**

RNA extraction, reverse transcription, qPCR, and RT-PCR analysis were performed in accordance with the work of Konishi et al. (2017). Plants were grown hydroponically in nutrient solution with 0.1 or 3 mM NH₄Cl and 10 µM KNO₃ for 6 weeks.

Gene-specific primers for GLN1;2, GLN1;3, GLN1;4, GLN2, and ubiquitin2 (UBQ2; AT2G36170) were prepared following the method of Konishi et al. (2017).

Serial fivefold dilution of cDNAs was used to calculate the standard curve and measure the amplification efficiency for each target and reference gene with LightCycler® 480 Software version 1.2.

**Western blotting**

Frozen whole-root samples were homogenized in four volumes of GS extraction buffer (Ishiyama et al. 2004b and Saito et al. 2017) using a mortar and pestle. The homogenates were centrifuged at 20,000 × g for 30 min at 4 °C. Soluble protein content was determined by the Bradford method (Bradford, 1976). Soluble proteins were separated by SDS-PAGE in a 12.5% (w/v) polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules) by electroblotting. Western blot analysis was carried out against 4 µg of total soluble protein extracted from the root. The membrane was incubated with anti-GS1 polyclonal antibody (Ishiyama et al. 2004b) and ECL Rabbit IgG (HRP-linked whole Ab from donkey; GE Healthcare Life Sciences, Marlborough). The ECL prime western blotting detection system (GE Healthcare Life Sciences, Marlborough) was used for detection. Signals were captured by an Image Quant 400 (GE Healthcare Life Sciences, Marlborough). The membrane was stained with MemCode™ Reversible Protein Stain as a loading control. Signal intensities were quantified with ImageJ (Abramoff et al. 2004).

**Nitrogen and carbon content**

Plants were grown hydroponically in a nutrient solution with 0.1 or 3 mM NH₄Cl and 10 µM KNO₃ for 6 weeks. Shoot or root samples were air-dried at 80 °C for 4–7 days and weighed with an electronic balance (XS Analytical Balances; Mettler-Toledo International Inc., Columbus). Dried samples were powdered with a Tissue Lyser II (Qiagen, K.K., Tokyo, Japan). Approximately 1-mg aliquots were wrapped in tin foil. The determination of total carbon and nitrogen contents was performed, as described previously (Konishi et al. 2017).
Measurement of free amino acids and ammonium

Plants were grown hydroponically in a nutrient solution with 0.1 or 3 mM NH₄Cl and 10 µM KNO₃ for 6 weeks. Free amino acids and ammonium were extracted from frozen shoot or root samples. The methods of extraction, purification, derivatization, and measurement were followed as described previously (Konishi et al. 2017).

Cellular localization of GLN1;1 and GLN1;4 promoter activities

The GLN1;1 promoter-GFP lines and GLN1;4 promoter-GFP lines originated from our previous study (Ishiyama et al. 2004b). Plants were grown in a hydroponic culture containing 0.1 or 3 mM ammonium and 10 µM nitrate as nitrogen sources for 6 weeks. Laser scanning confocal microscopy was performed with a Nikon C1si system. Details are as described previously (Konishi et al. 2017).

Xylem sap collection

Plants were grown hydroponically in nutrient solutions for 42 days (Loqué et al. 2006) and transferred to hydroponic solutions containing 0.1 or 3 mM ammonium and 10 µM nitrate as nitrogen sources. The hypocotyls were excised with a razor (Feather Safety Razor Co., Ltd., Osaka, Japan) at 3 days after transfer. Bleeding sap was then collected in a mounted silicon tube (internal diameter 1 or 1.5 mm; Asone Corporation, Osaka, Japan) for 20–30 min. Xylem collection started at 3 h after the light period. Xylem sap was stored at −80 °C. The concentrations of amino acids and ammonium were determined by UPLC (Nihon Waters K.K., Tokyo, Japan).

Statistics

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

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Disclosures

Conflicts of interest: No conflicts of interest are declared.

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References


Guan, M., de Bang, T.C., Pedersen, C. and Schjørring, J.K. (2016) Cytosolic glutamine synthetase Gln1;2 is the main isozyme contributing to GS1 activity in Arabidopsis shoots and can be up-regulated to relieve ammonium toxicity. Plant Physiol. 171: 1921-1933.


Krapp, A., Berthome, R., Orsel,


