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

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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 (Kiyomiya 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, GLN1;2, and GLN1;3 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 low-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 *GLN1*s were detectable in the *tko* (Fig. 3a).

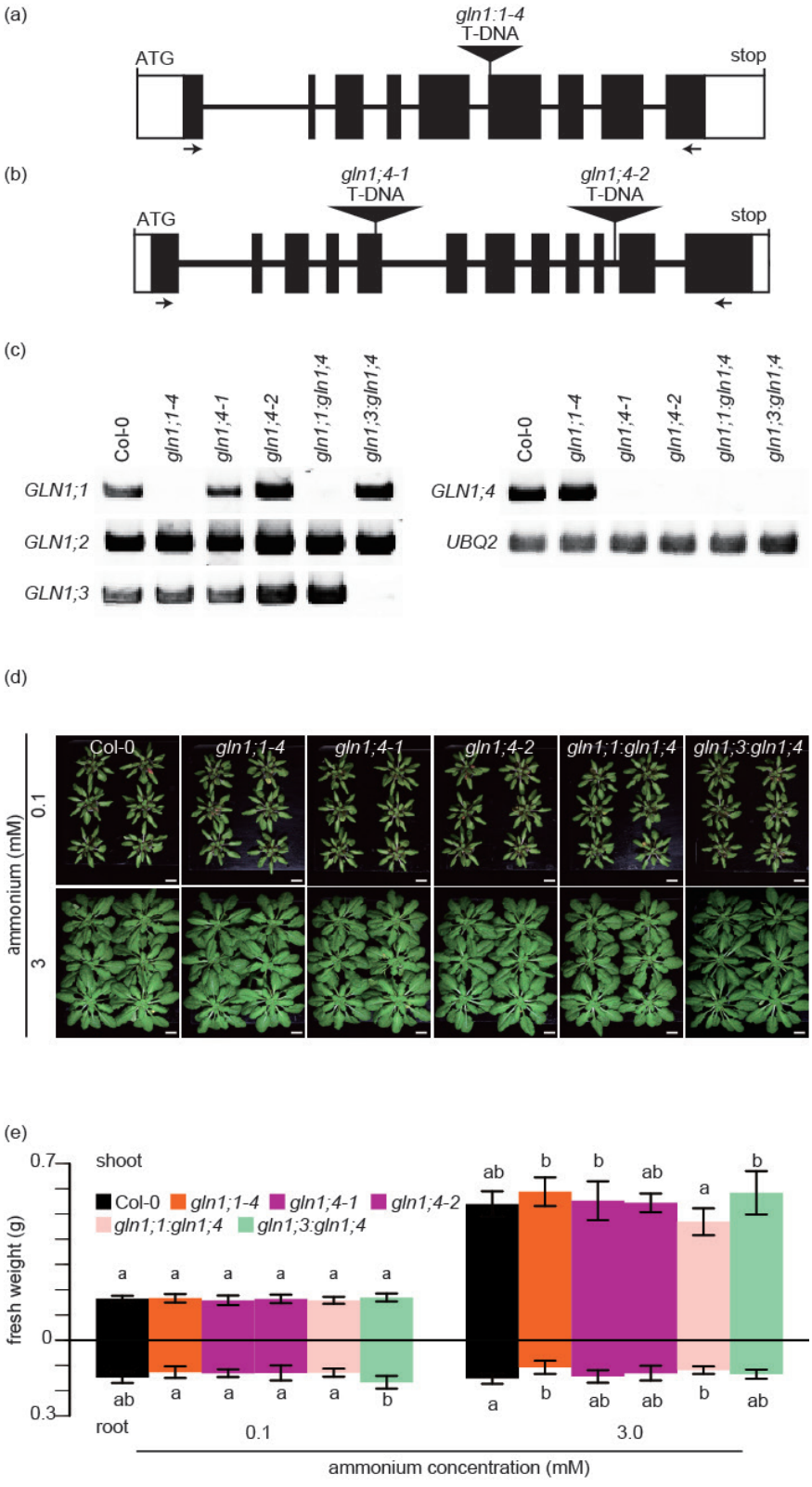


Figure 1. The *gln1;1 gln1;4* double knockout line does not show growth inhibition under the low-ammonium condition

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.

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

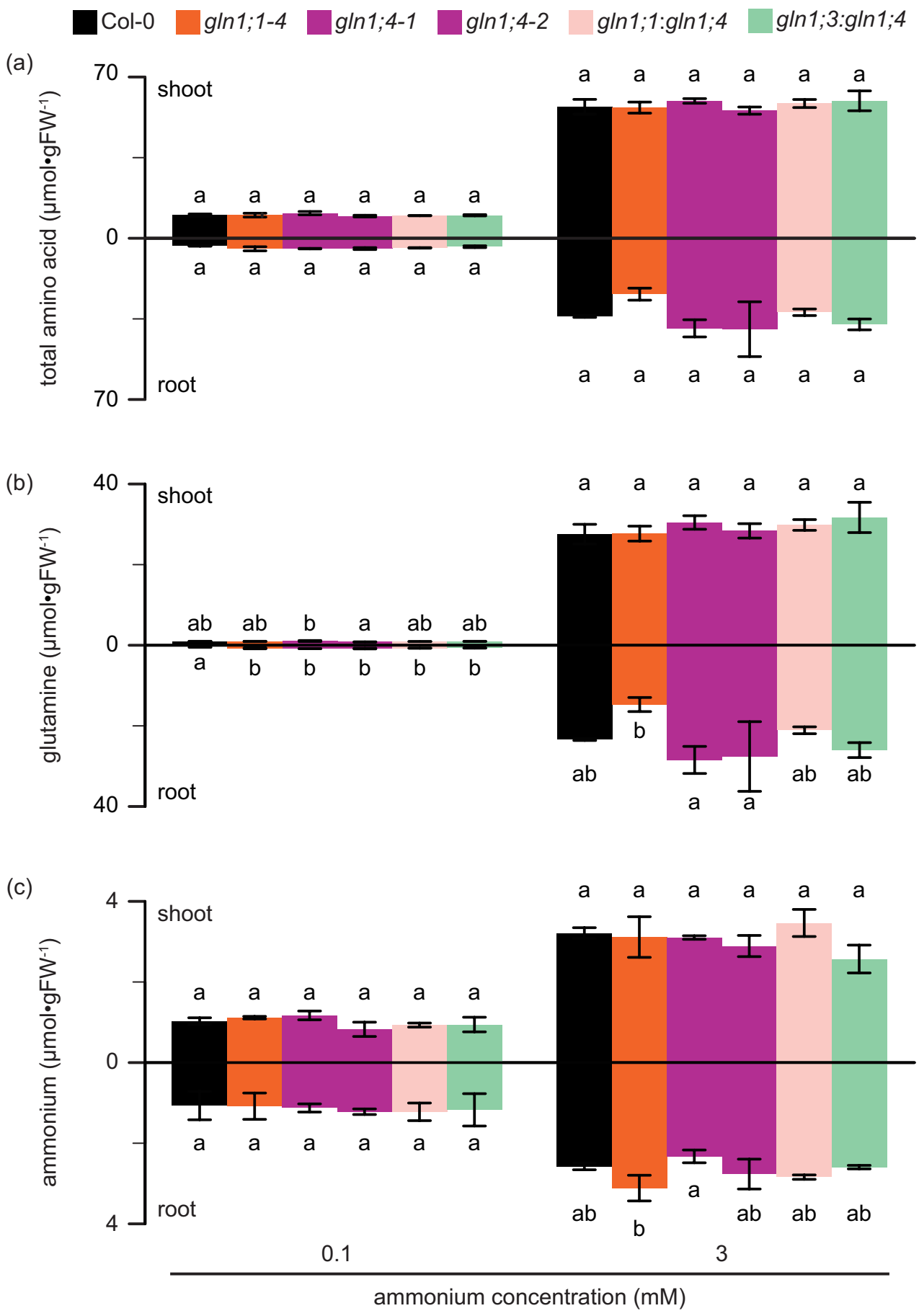

Col-0
gln1;1-4
gln1;4-1
gln1;4-2
gln1;1:gln1;4
gln1;3:gln1;4
(a)
total amino acid (µmol•gFW-1)
70
0
70
shoot
root
(b)
glutamine (µmol•gFW-1)
40
0
40
shoot
root
(c)
ammonium (µmol•gFW-1)
4
0
4
shoot
root
0.1
3
ammonium concentration (mM)

Figure 2. Ammonium and amino acid concentrations do not depend on *gln1;1 gln1;4* double knockout

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 (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). Plants were grown hydroponically for 6 weeks, supplemented with 0.1 or 3 mM ammonium and 10 µM nitrate as major nitrogen sources. Bars indicate mean ± standard deviation (SD) (n = 3). 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.

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 g*ln1;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

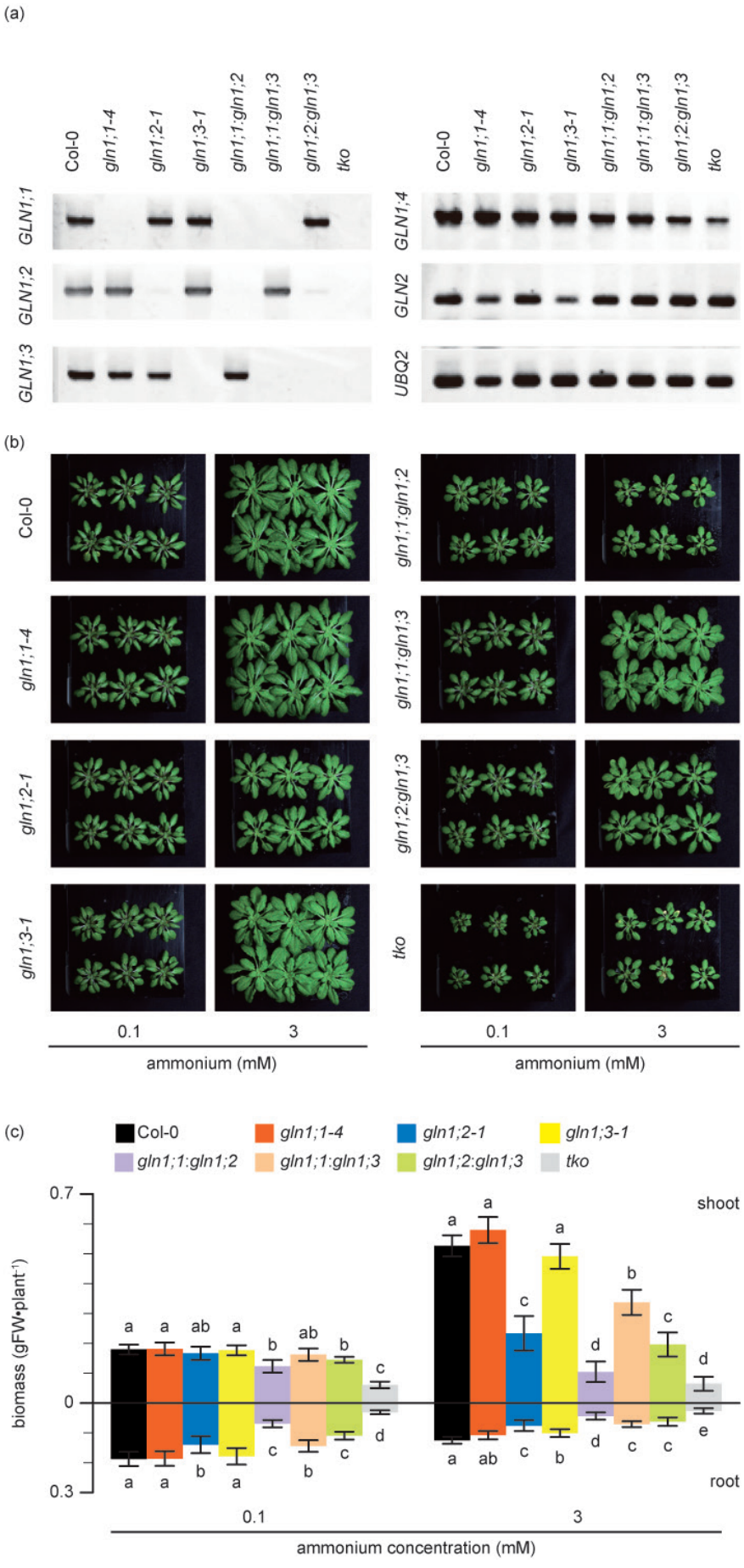


Figure 3. Knockout of *GLN1;2* and *GLN1;3* revealed the contribution of *GLN1;1* to ammonium assimilation in roots under nitrogen-sufficient and nitrogen-deficient growth conditions

(a) RT-PCR analysis of root RNA from wild type (Col-0) and *gln1;1*, *gln1;2*, and *gln1;3* mutants. Plants were grown hydroponically, supplemented with 0.1 mM ammonium as the major nitrogen source for 6 weeks. (b) Images and (c) fresh weight of shoots and roots of wild type (Col-0, black column), *GLN1* single mutants (*gln1;1*, orange-red column; *gln1;2*, blue column; and *gln1;3*, yellow column), double mutants (*gln1;1:gln1;2*, pale blue column; *gln1;1:gln1;3*, light orange column; and *gln1;2:gln1;3*, vivid green column), and triple mutant (*gln1;1:gln1;2:gln1;3*, light grayish cyan column). Plants were grown in hydroponic culture with 0.1 or 3 mM ammonium and 10 µM nitrate for 6 weeks. Data are calculated as mean ± SD (n = 12). One-way ANOVA, followed by Bonferroni test, was used, and significant differences at $p < 0.05$ within each group are indicated by different letters.

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*,

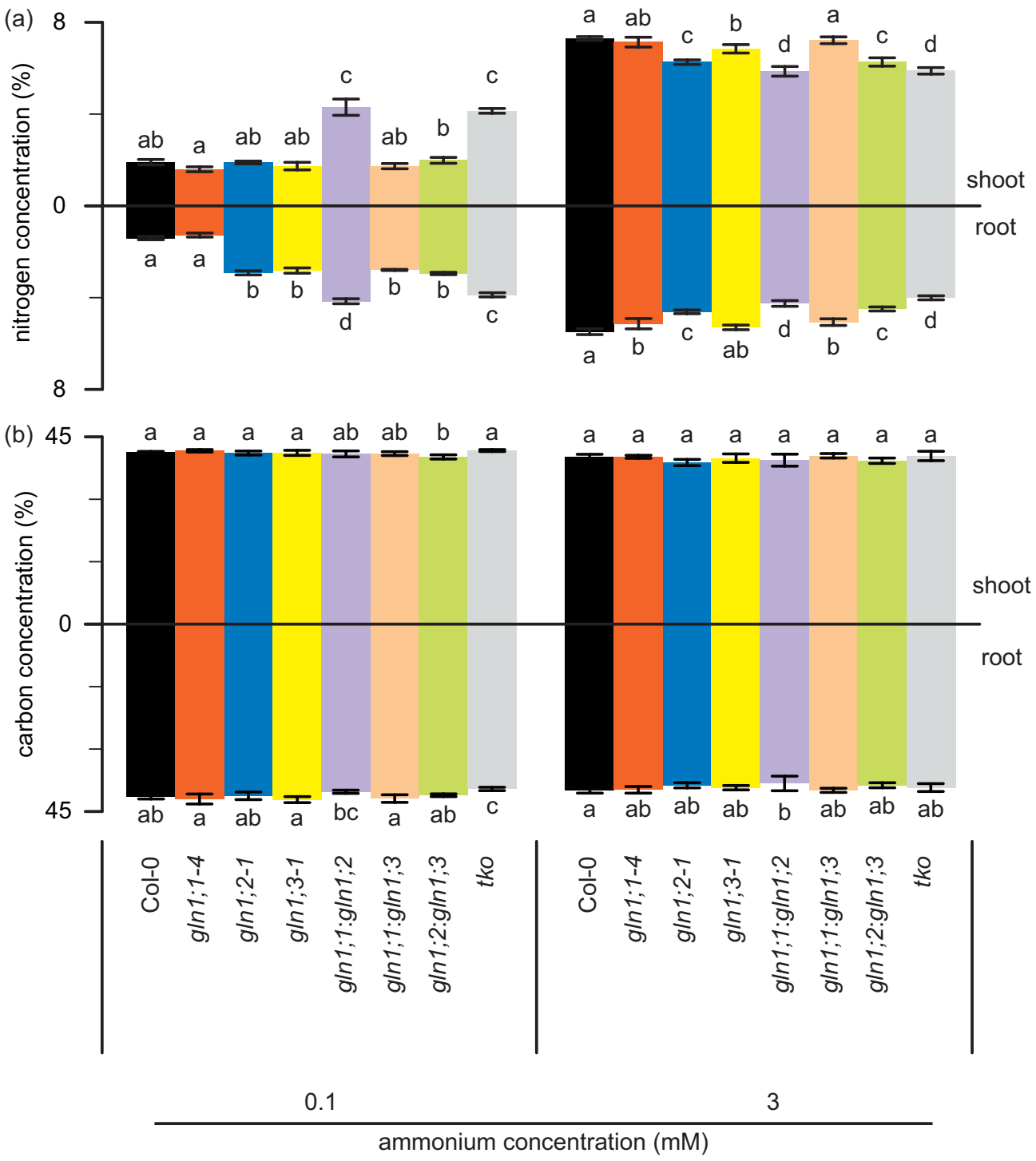


Figure 4. Ammonium-dependent nitrogen and carbon concentrations in roots and shoots of wild type and gln1 mutants

Nitrogen (a) and carbon (b) concentrations in shoots and roots of the wild type (Col-0), *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 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.

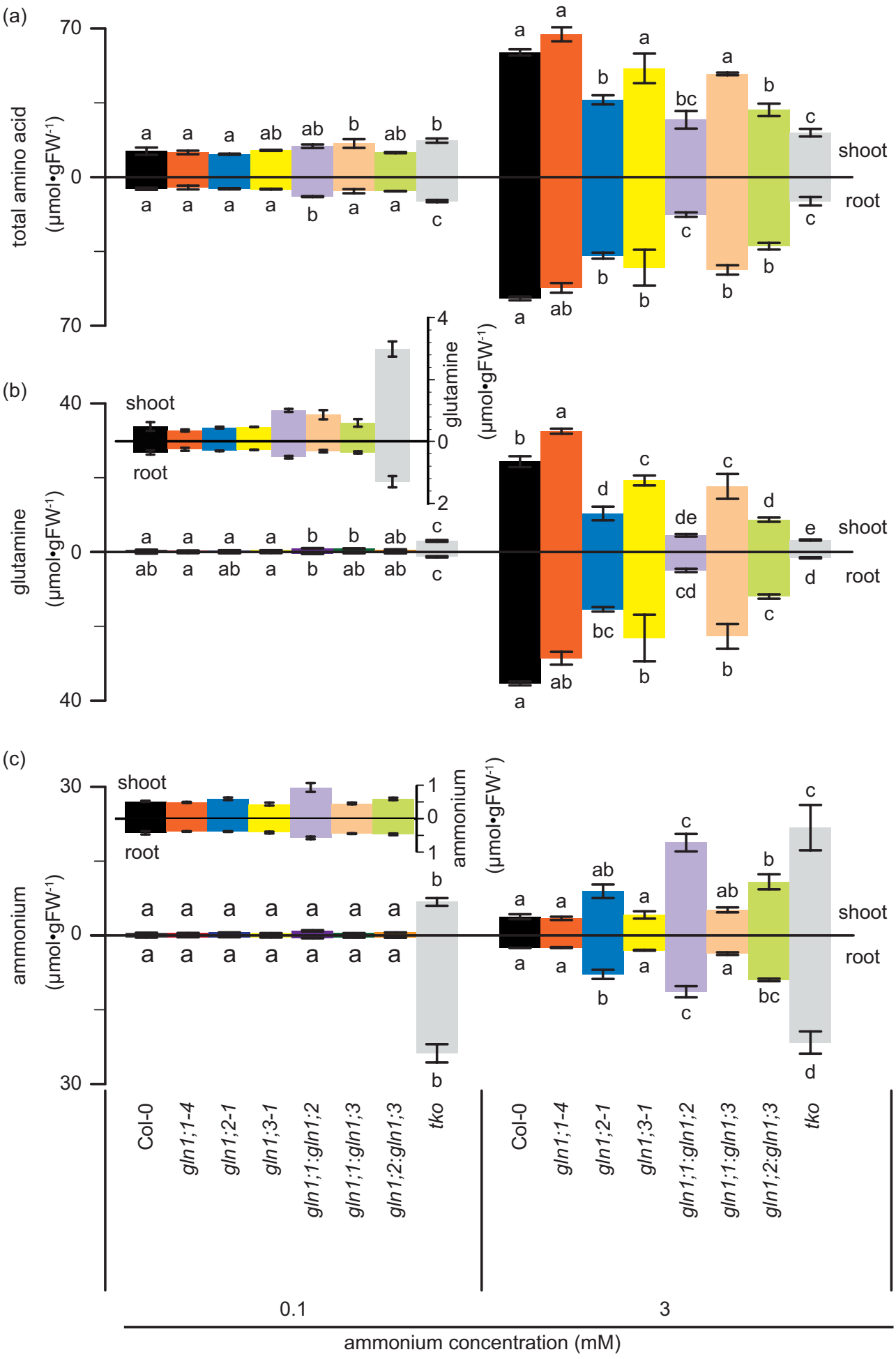

(a)
total amino acid (µmol•gFW-1)
70
0
70
shoot
root
(b)
glutamine (µmol•gFW-1)
40
0
40
shoot
root
glutamine (µmol•gFW-1)
4
0
2
(c)
ammonium (µmol•gFW-1)
30
0
30
shoot
root
ammonium (µmol•gFW-1)
1
0
1
Col-0
gln1;1-4
gln1;2-1
gln1;3-1
gln1;1:gln1;2
gln1;1:gln1;3
gln1;2:gln1;3
tko
0.1
3
ammonium concentration (mM)

Figure 5. Triple knockout in *GLN1* led to a marked accumulation of ammonium

The concentrations of total amino acids (a), glutamine (b), and ammonium (c) were measured in shoots and roots of the 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 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 = 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.

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 and 3 mM ammonium to evaluate the effect of the knockout of *GLN1* genes. Under the condition with 0.1 mM ammonium, the nitrogen concentration in roots of mutants, except *gln1;1*, was higher than that of the wild type. In particular, the nitrogen concentrations of *gln1;1:gln1;2* and tko were not only higher in roots but also in shoots (Fig. 4a).

The nitrogen might be concentrated in mutants, since their biomass was reduced. Under the condition with 3 mM ammonium, the nitrogen concentration was markedly diminished in the mutants with knockout of *GLN1;2*. Specifically, in the single mutant *gln1;2*, the double mutants *gln1;1:gln1;2* and *gln1;2:gln1;3*, and the triple mutant *tko*, there were significant decreases in nitrogen concentration in all cases (Fig. 4a). The nitrogen concentration in those mutants was approximately 15%–25% less than that of the wild type. Moreover, the single mutant *gln1;1* and the double mutant *gln1;1:gln1;3* showed significant reductions in nitrogen concentration in the roots (Fig. 4a). In contrast to the findings for the nitrogen concentration, there were few significant differences in the carbon concentration between the wild type and *gln1* mutants under both low- and high-ammonium conditions (Fig. 4b).

To investigate the contribution of individual *GLN1*s 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*,

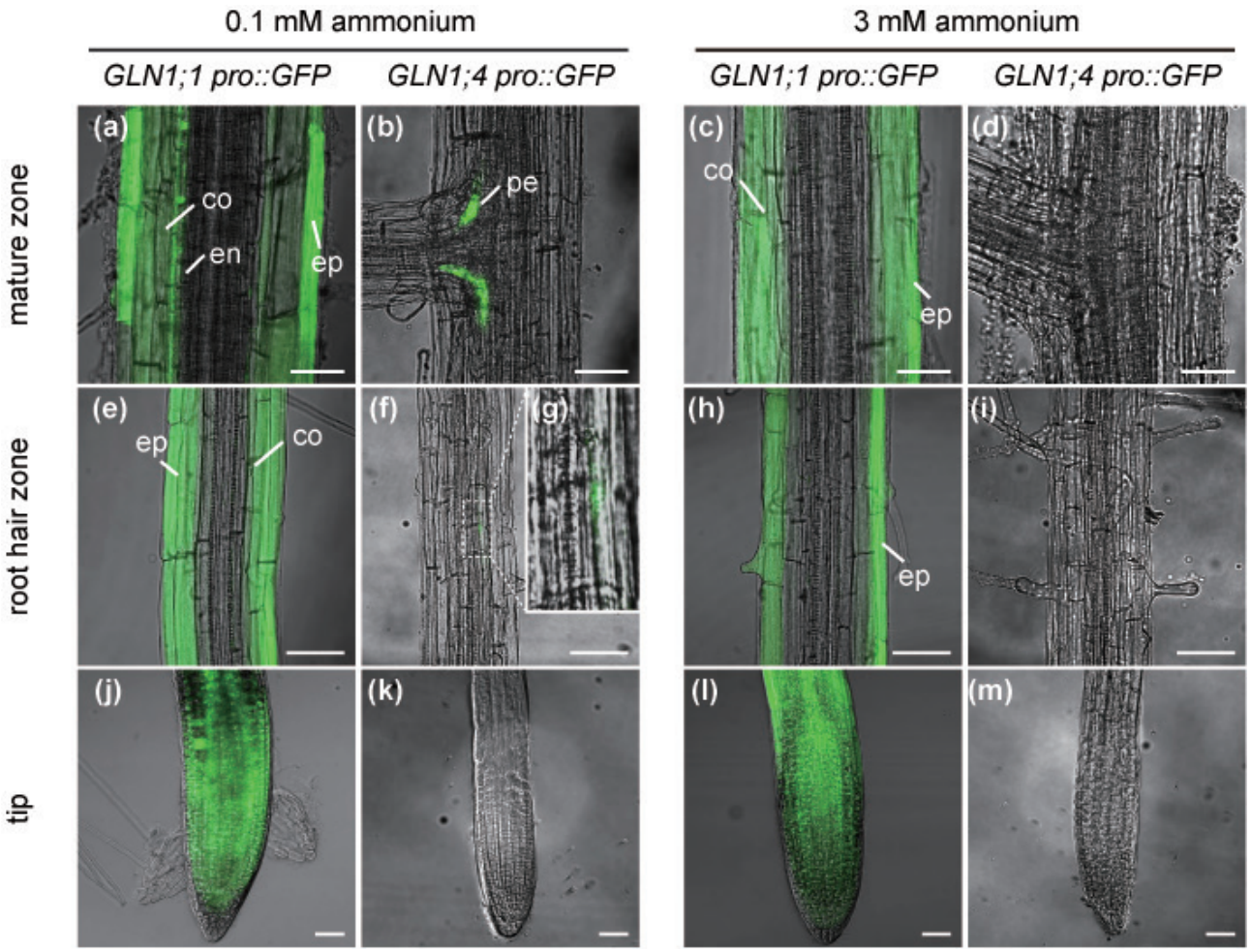


Figure 6. Localization of *GLN1;1* and *GLN1;4* promoter activities in roots

Transgenic plants expressing either the *GLN1;1* promoter:*GFP* (a, c, e, h, j, and l) or the *GLN1:4* promoter:*GFP* (b, d, f, g, i, k, and m) fusion gene constructs were grown for 6 weeks in hydroponic nutrient solutions containing either 0.1 mM (a, b, e, f, g, j, and k) or 3 mM ammonium (c, d, h, i, l, and m) and 10 μM nitrate as the nitrogen sources. Whole-mount images from root tips (j–m), root hair zones (e–i), and mature parts (a–d) were taken by confocal laser scanning microscopy. Boxed areas in (f) are magnified four times in (g). ep, epidermis; co, cortex; en, endodermis, pe, pericycle. Scale bars represent 50 μm.

also accumulated glutamine (Fig. 5a and 5b).

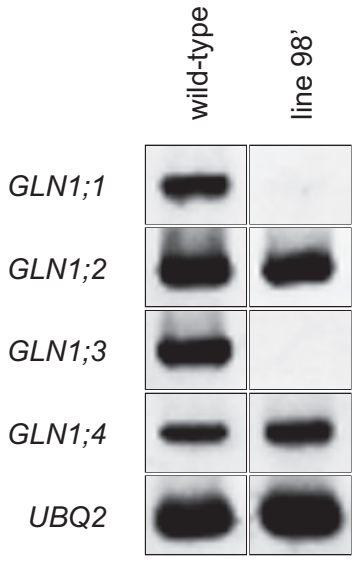
This accumulation of glutamine in tko might reflect its ammonium accumulation. Indeed, the ammonium concentration of tko was considerably increased, whereas gln1 single and double mutants did not show any differences from the wild type (Fig. 5c). The ammonium concentration of tko was 60 times higher 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

(a)

GLN1;4 \ GLN1;2	+/+	+/-	-/-
+/+	0	5	41
+/-	3	78	0
-/-	0	4	0

total 182 plants

(b)



(c)

GLN1;4 \ GLN1;2	+/+	+/-	-/-
+/+	0	7	73
+/-	3	99	1
-/-	0	0	0

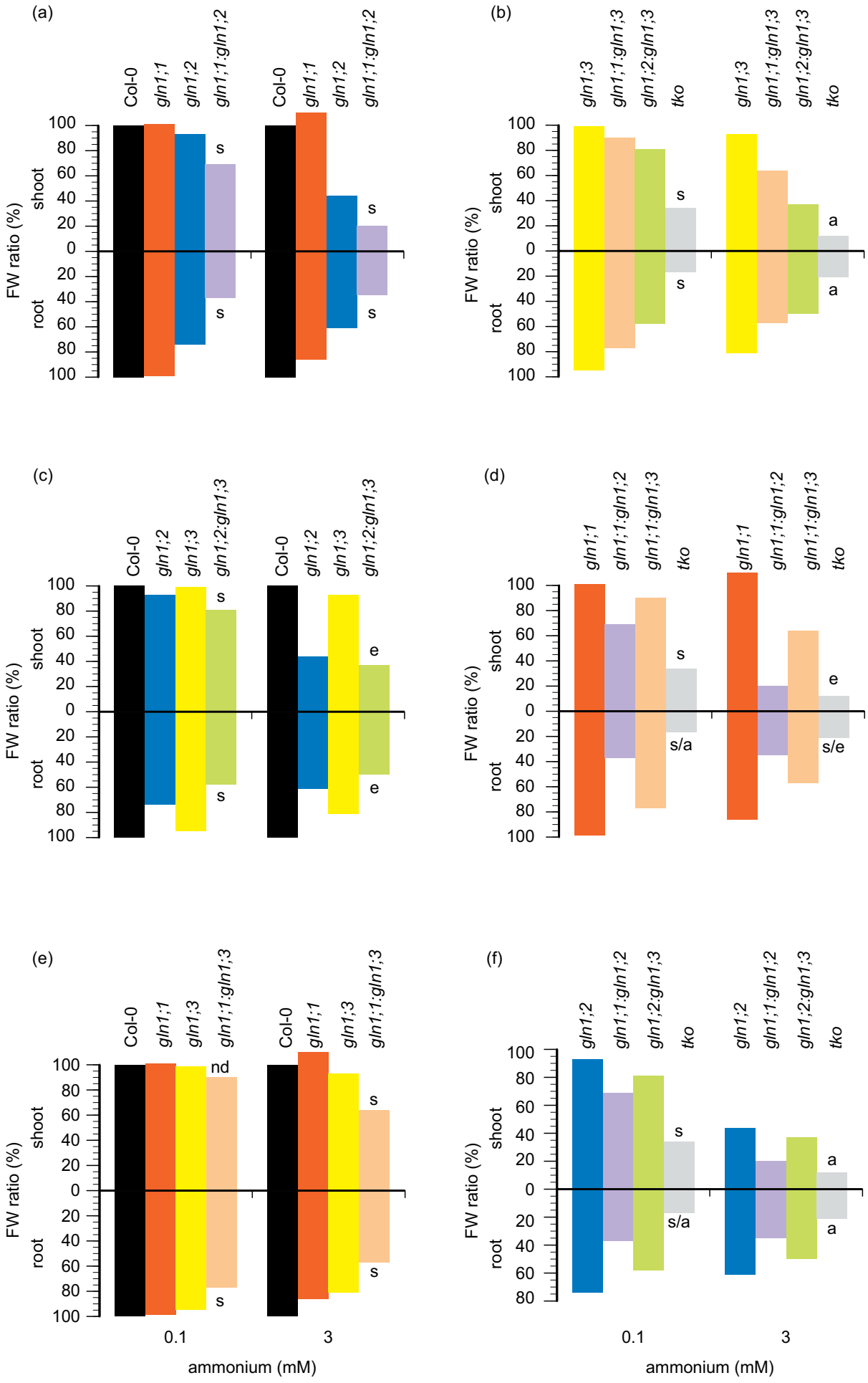
total 183 plants

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*.

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


(a)
Col-0
gln1;1
gln1;2
gln1;1:gln1;2
Col-0
gln1;1
gln1;2
gln1;1:gln1;2
FW ratio (%)
shoot
root
100 80 60 40 20 0 20 40 60 80 100
s
s
s
s
(b)
gln1;3
gln1;1:gln1;3
gln1;2:gln1;3
tko
gln1;3
gln1;1:gln1;3
gln1;2:gln1;3
tko
FW ratio (%)
shoot
root
s
s
a
a
(c)
Col-0
gln1;2
gln1;3
gln1;2:gln1;3
Col-0
gln1;2
gln1;3
gln1;2:gln1;3
FW ratio (%)
shoot
root
s
s
e
e
(d)
gln1;1
gln1;1:gln1;2
gln1;1:gln1;3
tko
gln1;1
gln1;1:gln1;2
gln1;1:gln1;3
tko
FW ratio (%)
shoot
root
s
s/a
e
s/e
(e)
Col-0
gln1;1
gln1;3
gln1;1:gln1;3
Col-0
gln1;1
gln1;3
gln1;1:gln1;3
FW ratio (%)
shoot
root
nd
s
s
s
0.1
3
ammonium (mM)
(f)
gln1;2
gln1;1:gln1;2
gln1;2:gln1;3
tko
gln1;2
gln1;1:gln1;2
gln1;2:gln1;3
tko
FW ratio (%)
shoot
root
s
s/a
a
a
0.1
3
ammonium (mM)

Supplemental Figure S2. Comparison of biomass in knockout lines for *GLN1*

The biomass data shown in Fig. 3 were arranged to interpret the synergistic interaction in double mutants. Plants were grown in a hydroponic culture with 0.1 or 3 mM ammonium and 10 μM nitrate for 6 weeks. The level of biomass relative to that in the wild type is shown. Comparison of single mutants and double mutants (a, c, and e), and comparison of double mutants and triple mutant (b, d, and f). The double mutant phenotype can be considered as additive (a), epistatic (e), or synergistic (s).

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 higher-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).

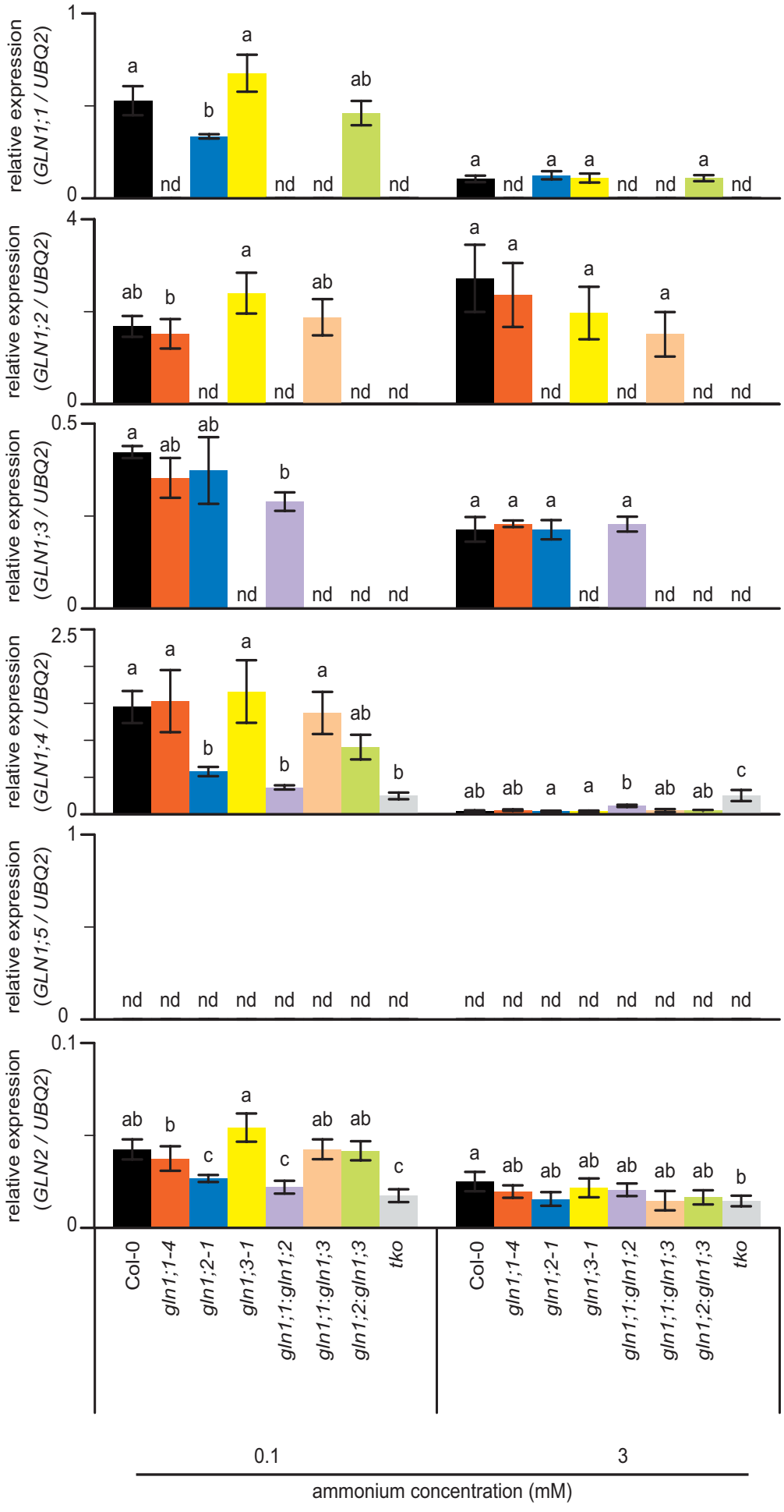
The promoter activities of GLN1;1 were localized in surface cell layers, and GLN1;4 was strongly expressed in the pericycle of basal parts of lateral roots

Figure 6 illustrates the localization of *GLN1;1* and *GN1;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). Under low-ammonium conditions, *GLN1;4* promoter activities were localized in the pericycle of basal regions of lateral roots (Fig. 6b) and were also observed in the pericycle of elongating regions (Fig. 6f and 6g). *GLN1;4*-dependent GFP signal was not detected at the root tip (Fig. 6k and 6 m). 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 S3). A previous biochemical study indicated that recombinant proteins of


relative expression (GLN1;1 / UBQ2)
relative expression (GLN1;2 / UBQ2)
relative expression (GLN1;3 / UBQ2)
relative expression (GLN1;4 / UBQ2)
relative expression (GLN1;5 / UBQ2)
relative expression (GLN2 / UBQ2)
Col-0
gln1;1-4
gln1;2-1
gln1;3-1
gln1;1:gln1;2
gln1;1:gln1;3
gln1;2:gln1;3
tko
0.1
3
ammonium concentration (mM)

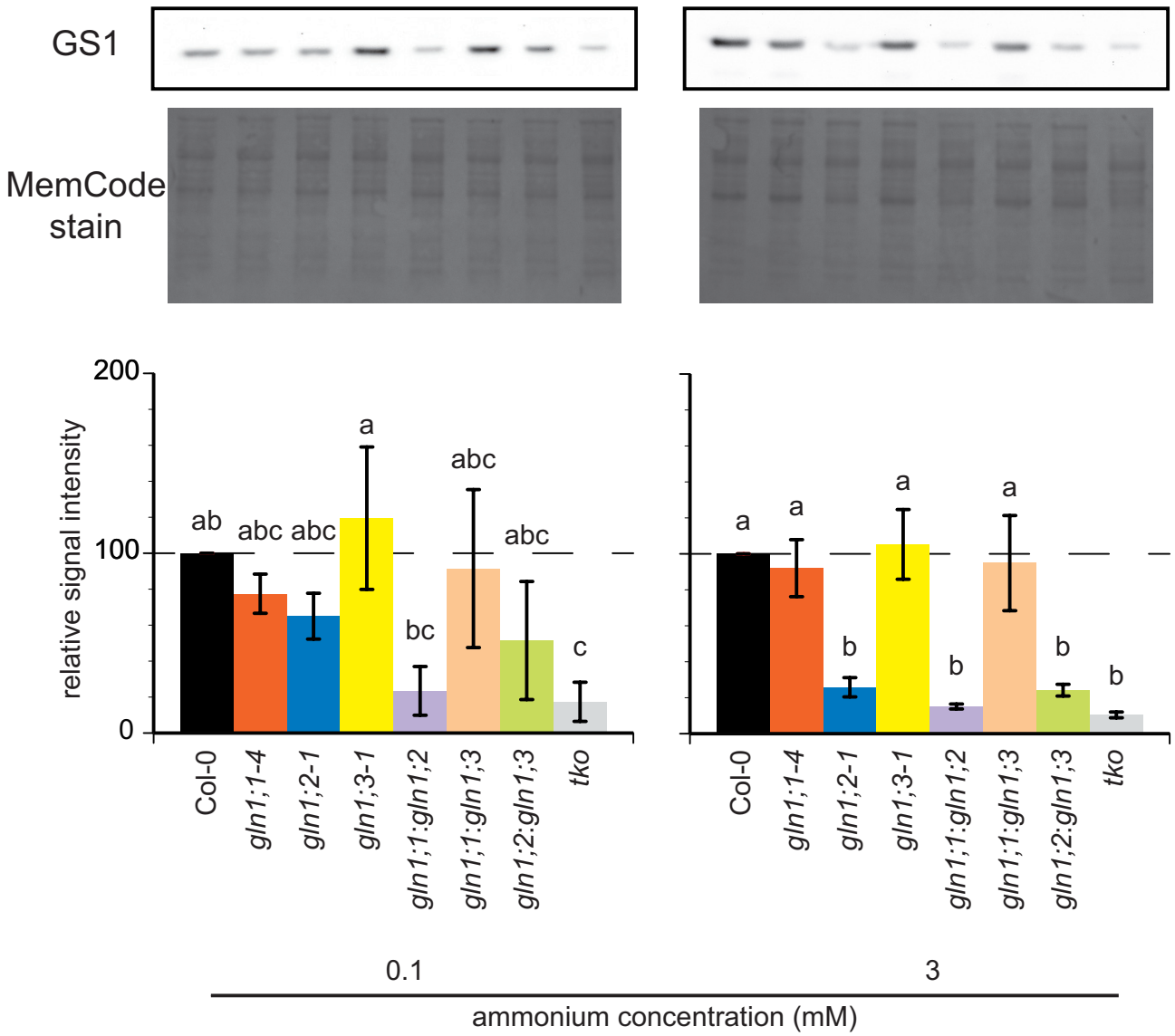
Supplemental Figure S3. Ammonium-dependent *GLN1* gene expression in roots of the triple mutant (tko)

GLN1;1, *GLN1;2*, *GLN1;3*, *GLN1;4*, *GLN1;5*, and *GLN2* mRNA abundance in roots of the wild type or mutants was quantified by qPCR. Plants were grown in hydroponic culture solution containing either 0.1 or 3 mM ammonium as the major nitrogen source for 6 weeks. *UBQ2* was used to standardize the signal intensity. Data are presented as mean ± SD (n = 3–4).

One-way ANOVA, followed by Bonferroni test, was used, and significant differences at $p < 0.05$ within each group are indicated by different letters. nd indicates not detectable.

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 (Bittsanszky 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).

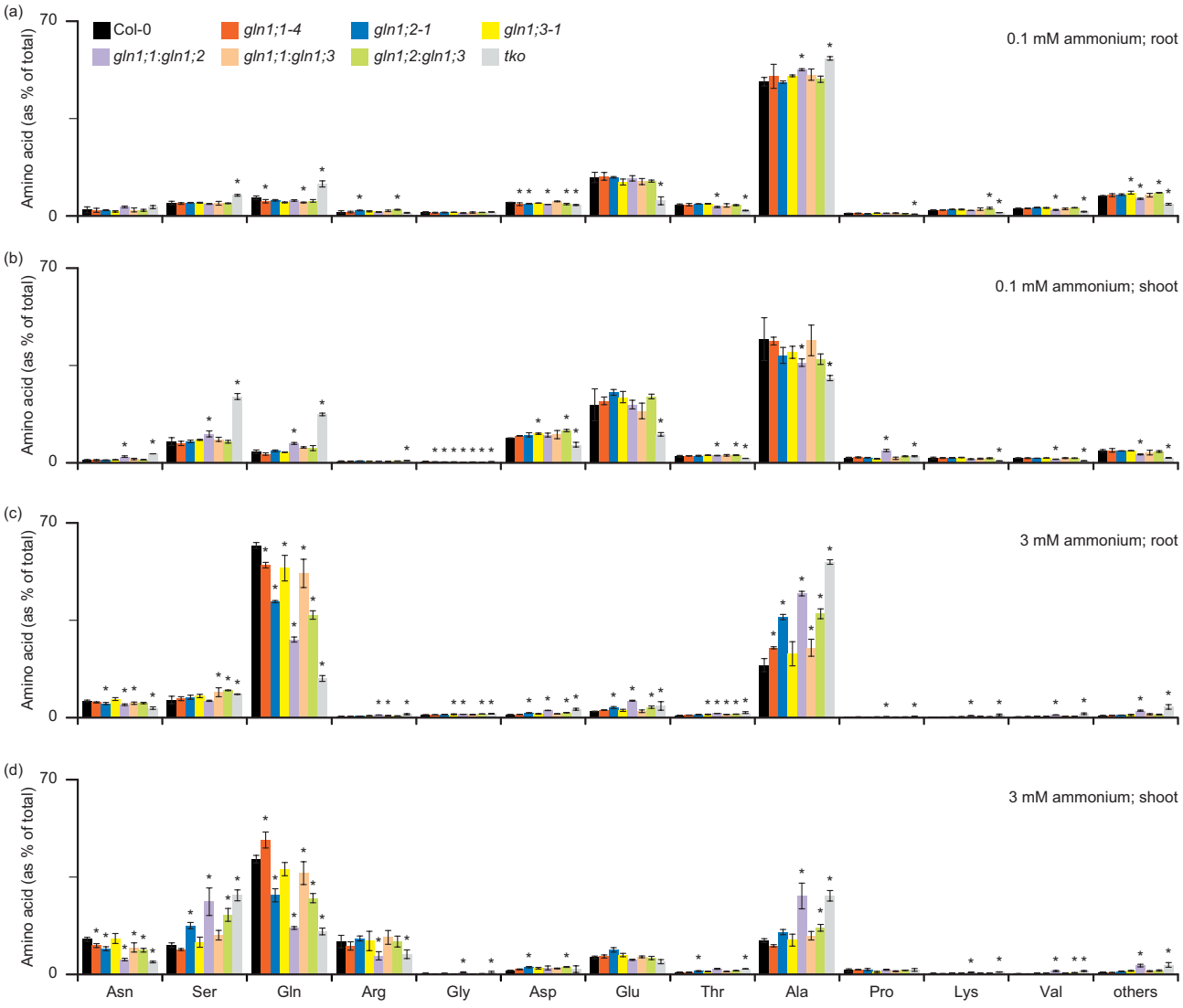


Supplemental Figure S4. The accumulation of GLN1 protein in roots of the triple mutant (tko)

The accumulation of GS1 protein in roots was determined using protein gel blot analysis. The protein concentrations were determined by the Bradford assay (Bradford 1976). Equal amounts of protein (4 µg) were separated by SDS-PAGE and blotted onto the membrane. Antibodies raised against recombinant rice GS1 were used for staining. The membrane was stained with MemCodeTM Reversible Protein Stain as a loading control. The analysis was repeated three times. The signal intensities were quantified using the software Image J (Abramoff et al. 2004). The signal intensities were standardized by that of the wild type.

One-way ANOVA, followed by Bonferroni test, was used, and significant differences at $p < 0.05$ within each group are indicated by different letters. nd indicates not detectable.

Triple mutations in *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

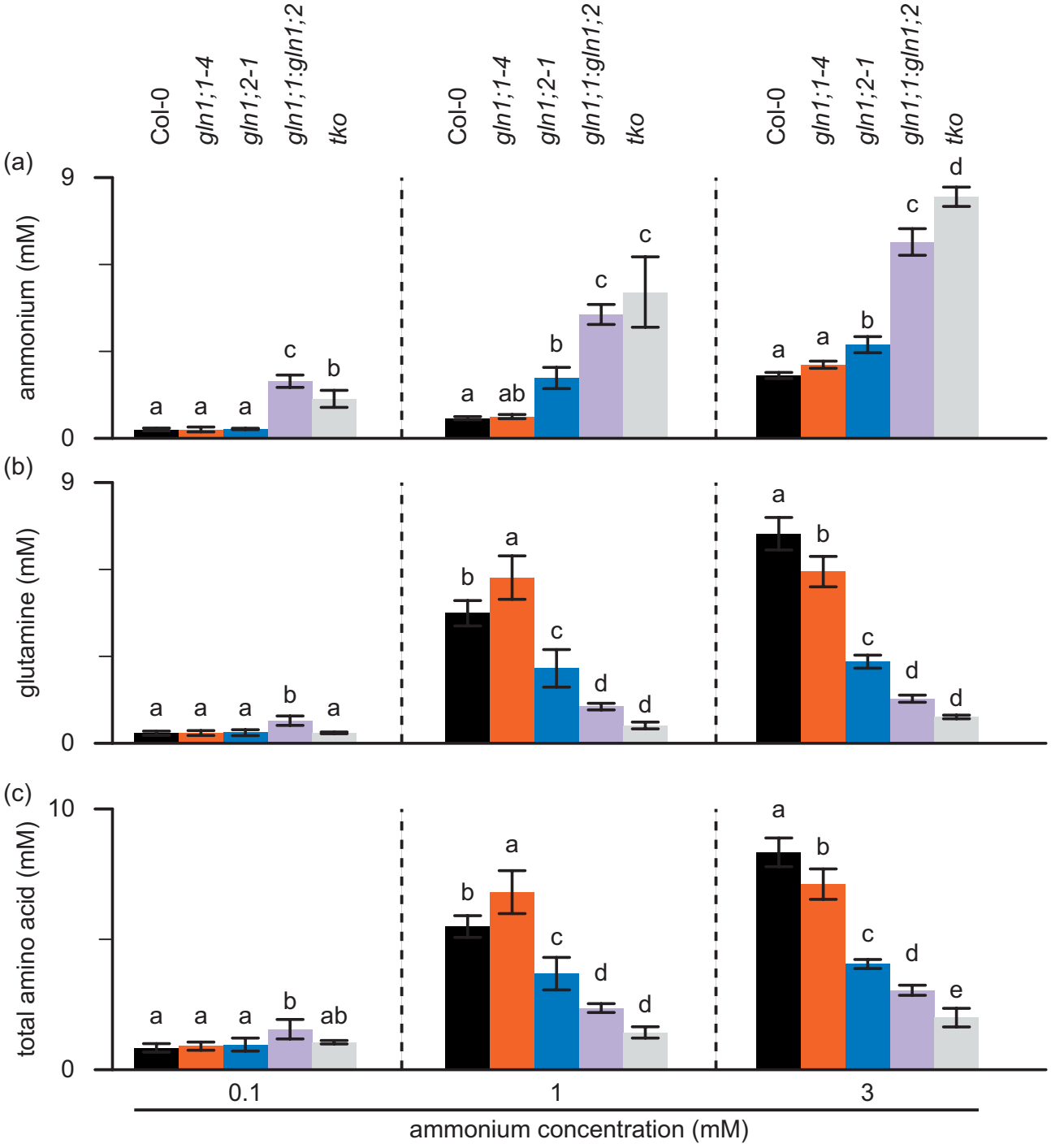


Supplemental Figure S5. Amino acid composition in shoots and roots of knockout lines for GLN1 under low- and high-ammonium conditions

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.

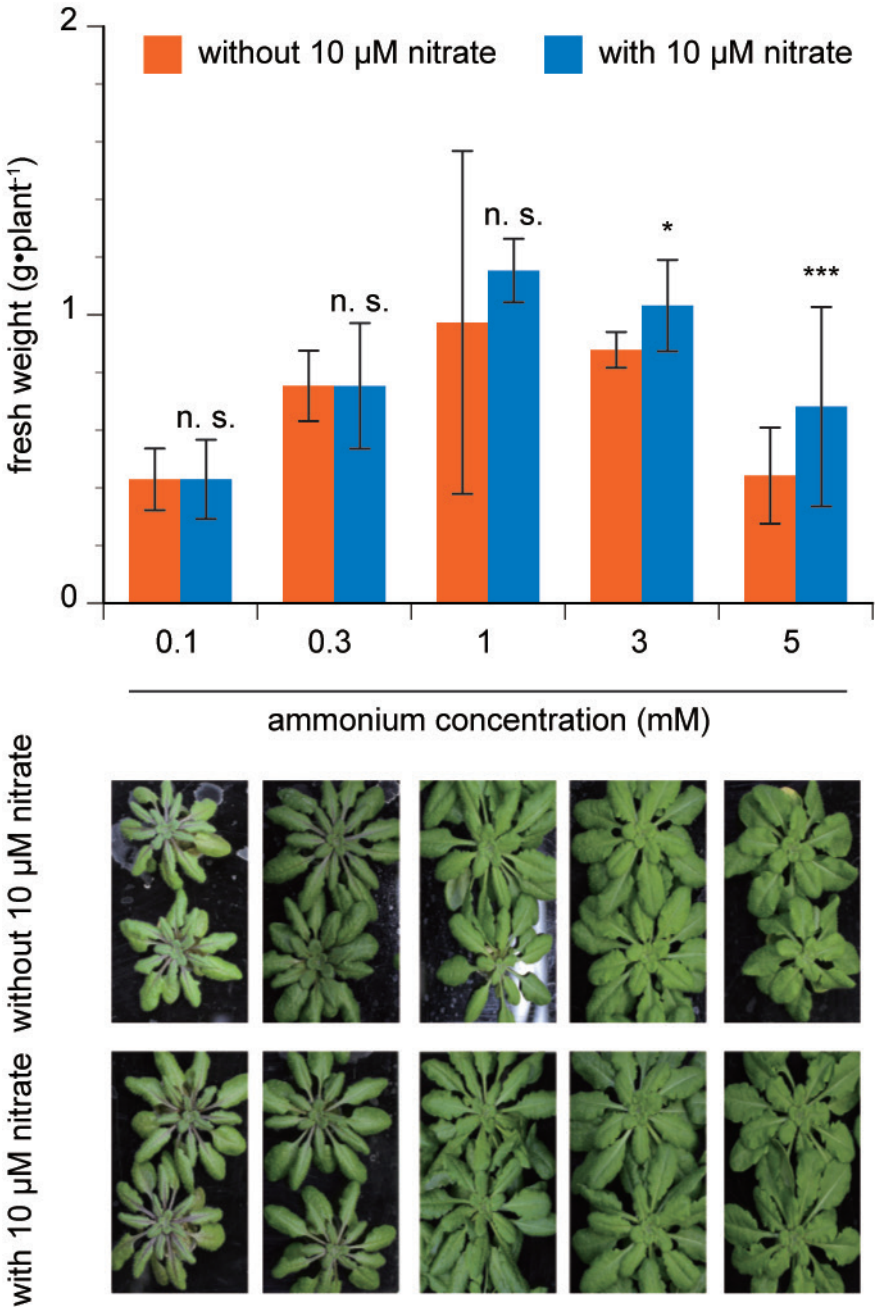
amino acids is dependent on the GS/GOGAT cycle. Because of the inhibition of ammonium assimilation in tko, plants might suffer from ammonium toxicity upon a low ammonium supply. Indeed, tko accumulated much more ammonium than the wild type (Fig. 5). At higher ammonium, the concentrations of Gln and Asn 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 was 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 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.



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 with asterisks: $*p < 0.05$ and $***p < 0.005$; n.s. indicates not significant.

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).

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

gln1:2 (+/-):*gln1;4* (+/-) and line 98' was not influenced, the segregation ratio did not follow Mendel's law of inheritance in both lines. The *P* values of the chi-square tests were 3.19×10^{-42} and 7.66×10^{-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.

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'- CAT CAA CCT TAA CCT CTC AGA CT - 3'*), *GLN1;1 cDNA R2* (*5'– TCT GCA ATC ATG GAA GTG ACA AT - 3'*), and *GABI T-DNA LB o8409* (*5'– ATA TTG ACC ATC ATA CTC ATT GC - 3'*); for *gln1;4-1*, *gln1;4 042546 F2* (*5' – GGT TTC GTA TAG AGC ATA AAG G - 3'*), *gln1;4 042546 R3* (*5' – TTT GAT CGC TGG GAG AAG TT - 3'*), and *T-DNA LB-01* (Konishi et al. 2017); and for *gln1;4-2*, *gln1;4 genome 117504 F* (*5'– GGA GTT GGA GCA GAC AAA GC - 3'*), *gln1;4 genome 117504 R* (*5'– AAG CTG GCC TAC GAT CTT CA - 3'*), and *T-DNA LB-01*.

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*; *gs1;2-1 -2 mutant genome F* (*5'– GGT TGG TGG TTC TGG TAT GGA CAT GAG – 3'*), *gs1;2-1 mutant genome R* (*5'– ACT TCA GCA ATA ACA TCA GGG TTA GCA – 3'*), *T-DNA LB-01*, *gln1;3-1*; *gln1;3-1 mutant genome F3* (*5'– CGC CGG TAT TGG TAT TTC TG – 3'*), *gln1;3-1 mutant genome R3* (*5'– CAG CTG AAG CTT CCC TAT CG – 3'*), *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 NH_4Cl and 10 μM KNO_3 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 NH_4NO_3 was removed to be replaced with 10 µM KNO_3 and various concentrations of NH_4Cl. 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 NH_4Cl and 10 µM KNO_3 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). For RT-PCR, GLN1;2-specific primers were *Gln1;2RF* (*5' – TGT TAA CCT TGA CAT CTC AGA CAA CAG T – 3'*, Ishiyama et al. 2004b) and *NK124* (*5' – CGG ATC ATC CTT TCA AGG GTT CCA GAG GAG – 3'*, Konishi et al. 2017).

The *GLN1;1*-specific primer pair was *gln11-4_qRTPCR_F3* (*5' – AAA CAA CAT TCT TGT CAT GTG C – 3'*) and *gln11-4_qRTPCR_R3* (*5' – GCA GGC CTT GTA GTG AGA ATC AAC AAC – 3'*).

For RT-PCR, the *GLN1;1*-specific primer pair was *GLN1;1 cDNA F2* (*5' – CAT CAA CCT TAA CCT CTC AGA CT – 3'*) and *GLN1;1 cDNA R2* (*5' – TCT GCA ATC ATG GAA GTG ACA AT – 3'*). For *GLN1;4*, the specific primer pair was *GS1;4F* (*5' – ATG TCT TCA CTT GCA GAT TTA ATC A – 3'*) and *GS1;4R* (*5' – TCA TGG TTT CCA AAG GAT TGT GGA T – 3'*). *GLN1;2*-, *GLN1;3*-, *GLN2*-, and *UBQ2*-specific primers 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 MemCodeTM (Thermo Scientific) 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 NH_4Cl and 10 µM KNO_3 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 weighed with an ultra-microbalance (UMX2; Mettler Toledo International Inc., Tokyo, Japan) and 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 NH_4Cl and 10 μM KNO_3 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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Conflicts of interest: No conflicts of interest are declared.

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