Cytosolic Glutamine Synthetase Gln1;2 Is the Main Isozyme Contributing to GS1 Activity and Can Be Up-Regulated to Relieve Ammonium Toxicity[OPEN]

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Cytosolic GS1 (Gln synthetase) is central for ammonium assimilation in plants. High ammonium treatment enhanced the expression of the GS1 isogene Gln-1;2 encoding a low-affinity high-capacity GS1 protein in Arabidopsis (Arabidopsis thaliana) shoots. Under the same conditions, the expression of the high-affinity low-capacity isoform Gln-1;1 was reduced. The expression of Gln-1;3 did not respond to ammonium treatment while Gln-1;4 and Gln-1;5 isogenes in all cases were expressed at a very low level. Gln-2 was highly expressed in shoots but only at a very low level in roots. To investigate the specific functions of the two isogenes Gln-1;1 and Gln-1;2 in shoots for ammonium detoxification, single and double knock-out mutants were grown under standard N supply or with high ammonium provision. Phenotypes of the single mutant gln1;1 were similar to the wild type, while growth of the gln1;2 single mutant and the gln1;1:gln1;2 double mutant was significantly impaired irrespective of N regime. GS1 activity was significantly reduced in both gln1;2 and gln1;1:gln1;2. Along with this, the ammonium content increased while that of Gln decreased, showing that Gln-1;2 was essential for ammonium assimilation and amino acid synthesis. We conclude that Gln-1;2 is the main isozyme contributing to shoot GS1 activity in vegetative growth stages and can be up-regulated to relieve ammonium toxicity. This reveals, to our knowledge, a novel shoot function of Gln-1;2 in Arabidopsis shoots.

Nitrate (NO₃⁻) and ammonium (NH₄⁺) are the major inorganic nitrogen (N) forms absorbed by plant roots (Yuan et al., 2013; Krapp et al., 2014; Krapp, 2015). Upon absorption, the inorganic N forms are assimilated into the low-molecular organic N compound Gln and subsequently incorporated into proteins, nucleic acids, and a range of secondary metabolites (Hawkesford et al., 2012). Gln synthetase (GS; EC 6.3.1.2) catalyzes the assimilation of ammonium into Gln and constitutes as such a central component of the N assimilatory pathway in plants (Bernard and Habash, 2009; Goodall et al., 2013; Yamaya and Kusano, 2014). N assimilation is compartmentalized in the cytosol and chloroplast. Correspondingly, two GS isoforms are encoded in the genome of higher plants: the cytosolic Gln synthetase isoform (GS1) and the chloroplastic Gln synthetase isoform (GS2). GS2 is encoded by a single gene (Gln-2), whereas GS1 is encoded by a multigene family, suggesting a complex role of GS1 with respect to plant N assimilation (Rodriguez et al., 2012). Arabidopsis (Arabidopsis thaliana) has five isogenes for GS1 (Gln-1;1–5) and the active structure of GS1 consists of octameric oligomers (Llorca et al., 2006). In roots, the individual GSI isoforms have specific localizations and expression patterns, as well as distinct kinetic properties with respect to ammonium and Glu (Guo et al., 2004; Ishiyama et al., 2004a, 2004b; Wang et al., 2013). Moreover, Gln-1;1 and Gln-1;2 were recently shown to play specific roles during seed germination and seed production (Guan et al., 2014). In particular, Gln-1;1 affected primary root development in response to exogenous N provision during seed germination, whereas Gln-1;2 was important for N remobilization in germinating seeds and for development of seed yield components (Guan et al., 2014). In maize (Zea mays), the isoform GSI-3 is present in the mesophyll cells, while GS1-4 is localized in bundle sheath cells. These two isoforms have different functions; GS1-3 primarily affects kernel number and
GS1-4 influences kernel size (Martin et al., 2006; Cañas et al., 2010). Knock-out of OsGS1;1 in rice (*Oryza sativa*) resulted in reduced growth and grain filling, while OsGS1;2 was critical for primary root NH$_4^+$ assimilation as well as in tillering (Funayama et al., 2013).

The expression levels of *Gln-1;1* and *Gln-1;2* in Arabidopsis are regulated in response to abiotic stress. Lothier et al. (2011) analyzed the *Gln-1;1* and *Gln-1;2* expression in the shoots of Arabidopsis grown at 2 mM (low) and 10 mM (high) nitrate. While the expression of *Gln-1;1* and *Gln-1;2* was similar at low nitrate, *Gln-1;1* was down-regulated and *Gln-1;2* was up-regulated at high nitrate. This differential response may be explained by different properties of Gln-1;1 and Gln-1;2. Gln-1;1 is a high-affinity isoform for ammonium with a $K_m < 10 \mu$M, while Gln-1;2 has a low affinity and high capacity ($K_m = 2450 \pm 150 \mu$M; Ishiyama et al., 2004b). Salt stress reduced *Gln-1;2* expression in Arabidopsis shoots, but at the same time enhanced the expression of both *Gln-1;1* and *Gln-1;2* in roots (Debouba et al., 2013). The role of the different GS1 isozymes in the ability of plants to cope with ammonium stress is not known.

We here hypothesize that individual GS1 isozymes play specific roles with respect to ammonium detoxification in Arabidopsis shoots. To test this, we characterized the single and double Arabidopsis knock-out mutants *gln1;1*, *gln1;2*, and *gln1;1:gln1;2* cultivated under both standard N (2 mM NH$_4$NO$_3$) and high (10 mM (NH$_4$)$_2$SO$_4$) ammonium conditions. Shoot growth, GS1 activity, and amino acid contents were sustained in *gln1;1* as compared to the wild type. In contrast, growth of the *Gln-1,2* knock-out mutants was markedly impaired.

**RESULTS**

*Gln-1;2* Expression in Shoots Is Up-Regulated by High Ammonium Supply

The expression levels of the five GS1 isogenes, *Gln-1;1-5*, and *Gln-2* were examined by RT-qPCR (Fig. 1). In the wildtype shoots, *Gln-2* expression was higher than that of the GS1 isogenes, showing that GS2 is the predominant GS isoform in leaves of vegetatively growing plants (Fig. 1A). In contrast, *Gln-2* expression...
was very low in roots (Fig. 1B). Compared with standard N conditions, $Gln-1;1$ expression was significantly down-regulated ($P \leq 0.01$), while $Gln-1;2$ was significantly up-regulated ($P \leq 0.01$; Fig. 1A) in shoots of plants receiving high ammonium supply. In roots, both $Gln-1;1$ and $Gln-1;2$ were up-regulated by high ammonium supply (Fig. 1B). The expression level of $Gln-1;3$ remained unchanged in both shoots and roots after ammonium treatment, and that of $Gln-1;4$ and $Gln-1;5$ were in all cases very low, irrespective of ammonium supply.

$Gln-1;2$ Is Essential for Plant Vegetative Growth and Ammonium Assimilation

To identify the specific roles of $Gln-1;1$ and $Gln-1;2$ in coping with high ammonium treatment, single and

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**Figure 2.** Characterization of Arabidopsis mutants $gln1;1$, $gln1;2$, and $gln1;1:gln1;2$ growing under standard N (gray bars) or high ammonium conditions (black bars). A, Images of $gln1;1$, $gln1;2$, $gln1;1:gln1;2$, and wild-type plants growing under standard N (top row) or high ammonium (bottom row) conditions. B, Shoot fresh weight of mutants and wild type ($n = 24$). C, Root fresh weight of mutants and wild type ($n = 24$). D, Shoot ammonium content in mutants and wild type ($n = 6$). The break on the y axis omits values between 3 and 6 $\mu$mol NH$_4^+$ g$^{-1}$ fresh weight. E, Root ammonium content in mutants and wild type ($n = 6$). Results are means ± se of six biological replicates with each replicate consisting of at least four plants. The symbols # and * indicate statistically significant differences between high ammonium and standard N conditions (#, $P \leq 0.05$ and **, $P \leq 0.01$) and between mutants and the wild type (*, $P \leq 0.05$ and ***, $P \leq 0.01$), respectively. FW, Fresh weight; HA, high ammonium; WT, wild type.
double mutant plants *gln1;1, gln1;2, and gln1;1:gln1;2* were characterized. Under standard N (2 mM NH₄NO₃) conditions, *gln1;1* plants appeared similar to wild-type plants, while *gln1;2* and *gln1;1:gln1;2* were markedly smaller (Fig. 2A). Visual symptoms of ammonium toxicity were also most severe in *gln1;2* and *gln1;1:gln1;2* plants (Fig. 2A). In wild-type and *gln1;1* plants, toxicity symptoms first appeared on the younger leaves where the margins became chlorotic and curled upwards. In *gln1;2* and *gln1;1:gln1;2* plants, mid-to-older leaves were also affected and in *gln1;1:gln1;2*, the chlorosis between the veins progressed to scattered necrotic spots (Fig. 2A).

The shoot fresh weight of plants in the high ammonium treatment was significantly lower (*P* ≤ 0.01) than that of plants growing at standard N supply (Fig. 2B). Under both standard N and high ammonium conditions, *gln1;2* and *gln1;1:gln1;2* plants showed a significant decrease (*P* ≤ 0.01) in shoot fresh weight compared to the wild type (Fig. 2B). A similar pattern was observed when comparing the root fresh weight of plants grown under standard N or high ammonium conditions (Fig. 2C). Also the root growth of *gln1;1* was significantly reduced (*P* ≤ 0.05) at high ammonium treatment (Fig. 2C).

Knock-out of Gln-1;1 and/or Gln-1;2 is expected to block Gln synthesis resulting in ammonium accumulation. This was indeed the case as shown by significantly higher ammonium content in both the shoots and roots of the *gln1;2* and *gln1;1:gln1;2* mutants compared to the wild type at standard N supply (Fig. 2, D and E). In contrast, knock-out of Gln-1;1 did not lead to an increase in tissue ammonium content under standard N supply (Fig. 2, D and E). High ammonium treatment caused elevated shoot contents of ammonium in wild type and all mutant plants (compare gray and black bars in Fig. 2D). Relative to the wild type, only the double mutant had significantly higher tissue content of ammonium under high ammonium supply (Fig. 2, D and E).

Expression of Gln-1;1, Gln-1;2, and Gln-1;3 in Mutant and Wild-Type Plants

The expression levels of the main GS1 isogenes Gln-1;1, Gln-1;2, and Gln-1;3 (Fig. 1) were analyzed by RT-qPCR in the shoots (Fig. 3, A–C) and roots of all mutant and wild-type plants growing under standard

![Figure 3](https://www.plantphysiol.org/)

**Figure 3.** Gln-1;1, Gln-1;2, Gln-1;3, and Gln-2 expression in the shoots of mutant and wild-type Arabidopsis plants growing under standard N (gray bars) or high ammonium (black bars) conditions. A, Gln-1;1. B, Gln-1;2. C, Gln-1;3. D, Gln-2. Values are fold changes in gene expression relative to Ref. 1 expression, determined from the CT values using the Pfaffl method (Pfaffl 2001), then normalized to the wild type in standard N conditions. Three technical repetitions were performed for each sample, and three biological replicates were included. Symbols # and * indicate statistically significant differences between high ammonium and standard N conditions (#, *P* ≤ 0.05 and **#, *P* ≤ 0.01) and between mutants and the wild type (*#, *P* ≤ 0.05 and ***, *P* ≤ 0.01), respectively. WT, Wild type.
N or high ammonium conditions (Supplemental Fig. S1). Glh-1;1 expression in wild-type shoots was decreased by high ammonium supply (Fig. 3A). In contrast, the transcript level of Glh-1;2 was significantly up-regulated (P ≤ 0.01) in wild type and gln1;1 (Fig. 3B). Glh-1;3 expression remained unchanged in shoots (Fig. 3C), but increased significantly (P ≤ 0.01) in roots of the double mutant gln1;1:gln1;2 under high ammonium conditions (Supplemental Fig. S1C). The expression of Glh-2 encoding chloroplastic GS increased (P ≤ 0.05) in the wild type but was markedly depressed (P ≤ 0.01; Fig. 3D) in the gln1;1:gln1;2 double mutant in response to high ammonium supply.

**Gln-1;2 Is Important in Contributing to GS1 Activity for N Assimilation**

In roots, GS is the main isoform for ammonium assimilation (Fig. 1). The observed increase in root ammonium content in the two Glh-1;2 knock-out mutants (Fig. 2E) was related to a significant reduction in the root GS activity under both standard N and high ammonium conditions (Fig. 4B). In contrast, the root GS activity in gln1;1 was not affected (Fig. 4B), consistent with the similar root ammonium contents in the wild type and gln1;1 under both growing conditions (Fig. 2E). In shoots, both GS1 and GS2 contribute to ammonium assimilation into Gln. We first assayed total GS activity in the shoots of mutants and wild-type plants (Fig. 4A) and then used ion-exchange chromatography to quantify the separate shoot GS1 and GS2 activities (Figs. 5 and 6) under both standard N and high ammonium conditions. No significant difference in the total GS activity, i.e., GS1 plus GS2 activity, was observed in the knock-out mutants compared to wild-type plants grown under standard N conditions (Fig. 4A). However, in the high ammonium-treated plants, a significant decrease (P ≤ 0.01) in the total GS activity was measured in gln1;1:gln1;2 (Fig. 4A). High ammonium treatment caused significantly (P ≤ 0.01) higher contents of total free amino acid and Gln in both wild-type and mutant plants (Fig. 4, C and D). Comparing wild-type and mutant lines, the content of total soluble amino acids did not change in gln1;1 but decreased significantly (P ≤ 0.05) in gln1;2 (Fig. 4C). The content of free Gln in shoot tissues of the gln1;1 mutant was maintained at the same level as in the wild-type under both growing conditions (Fig. 4D). In contrast, a significant decrease (P ≤ 0.01) in the Gln content occurred in gln1;2 and gln1;1:gln1;2, especially at high ammonium treatment, showing an important role of Glh-1;2 with respect to ammonium
detoxification by assimilation of ammonium into Gln in vegetative leaves (Fig. 4D).

Following ion-exchange chromatography to separate GS1 and GS2 protein, three peaks of GS activity were detected in wild-type plants (Figs. 5A and 6A). Western-blot analysis showed that the first two GS peaks contained GS2, whereas the third peak contained GS1 (Fig. 5A). To examine whether the separate GS2 activity peaks contained different enzymatically active oligomers, we further performed Blue Native-PAGE with non-denatured protein fractions obtained by HPLC separation, followed by immunoblot analysis (Fig. 5B). Total leaf soluble proteins, not separated by HPLC, were loaded on the gel (lanes 1 and 2) to identify GS enzymatic oligomers eluting in the HPLC fractions. Lanes 3 to 15 correspondingly contained native proteins from fractions 3 to 15. In total, five different GS bands were detected in the leaf-soluble protein fractions using immunoblotting (lanes 1 and 2). The first GS2 activity peak (lanes 4 and 5) contained two of the GS bands, whereas two additional bands of higher $M_r$ were detected in the second GS2 activity peak (lanes 6–9; Fig. 5B). The intensity of the GS2 bands corresponded to the western-blot analysis (shown below Fig. 5A) and the GS activity in the HPLC fractions (Fig. 5A). The GS1 activity peak also contained two GS bands (lanes 12–14) of which one differed from the GS2 bands (Fig. 5, A and B).

In gln1;1, GS1 activity did not decrease in comparison to the wild type (fractions 12–14; Figs. 5C and 6B). However, in gln1;2 and gln1;1:gln1;2, the GS1 peak was not detected (Figs. 5, D and E, and 6, C and D). This suggests that Gln-1;2 was the main isozyme contributing to shoot GS1 activity in vegetative growth stages and corresponded with the fact that Gln-1;2 was able to be up-regulated in shoots to rescue the plants from ammonium toxicity.

**GS1 Protein Content Is Increased after High Ammonium Treatment**

Quantitative protein analysis by laser-ablation inductively coupled plasma mass spectrometry (LA-ICP-MS)
showed that total GS1 was maintained at control levels in gln1;1, while being markedly reduced in gln1;2 and gln1;1:gln1;2 (Fig. 7A). The analysis of GS1 protein content also revealed that GS1 levels increased under high ammonium conditions in both wild-type plants and mutants (Fig. 7A). No significant difference in GS2 content was observed at the protein level using LA-ICP-MS to quantify GS2 abundance (Fig. 7B).

**DISCUSSION**

Two Arabidopsis T-DNA insertion lines for the cytosolic GS1 isogenes *Gln-1;1* and *Gln-1;2* were grown together with their corresponding double mutant at standard (2 mM NH$_4$NO$_3$) versus high (10 mM (NH$_4$)$_2$SO$_4$) ammonium supply. To alleviate toxicity, plants exposed to high levels of ammonium reduce the transport of ammonium to the shoots by increasing ammonium assimilation in the roots. GS1 consequently plays an important role in ammonium tolerance (Sarasketa et al., 2014; Zhou et al., 2015). However, ammonium translocation to the shoot will inevitably increase (Finnemann and Schjoerring, 1999; Schjoerring et al., 2002). Arabidopsis shoots are believed to be significantly more sensitive to ammonium stress than roots (Li et al., 2014). In this work, Arabidopsis plants exposed to the high ammonium treatment developed clear ammonium toxicity symptoms in the form of leaf chlorosis, reduced plant growth, increased tissue ammonium content, and changed amino acid composition (Figs. 2 and 4; see also Sarasketa et al., 2014). The most severe ammonium toxicity phenotype was shown by the *gln1;2* and *gln1;1:gln1;2* knock-out lines (Fig. 2A). Even in the absence of ammonium toxicity, excessive ammonium accumulation (Fig. 2, D and E) and a depression of plant growth (Fig. 2, A–C) occurred in the *gln1;2* and *gln1;1:gln1;2* mutants. This shows that...
Gln-1;2 plays a role of so far unknown importance with respect to ammonium assimilation.

Expression of GS1 Isoforms in Arabidopsis Shoots in Relation to Ammonium Detoxification

Evidence for a special role of Gln-1;2 in alleviation of ammonium toxicity is provided by the significant up-regulation of Gln-1;2 expression under high ammonium conditions. The up-regulation was observed both in wild-type plants (Figs. 1A and 3B) and, even more pronounced, in the gln1;1 single knock-out mutant (Fig. 3B). This allowed the gln1;1 mutant to cope with ammonium stress (Fig. 2, A, B, and D) by maintaining total GS1 protein abundance and activity (Figs. 6, A and B, and 7A), as well as free amino acid and Gln contents at wild-type levels in the shoots (Fig. 4, C and D). Gln-1;2 has a low affinity toward ammonium (Ishiyama et al., 2004b) and is able to operate with high capacity at the relatively high levels of ammonium encountered in shoot tissues (2–20 mM). In contrast, Gln-1;1 has a high affinity toward ammonium (Ishiyama et al., 2004b) and will have a relatively limited maximal capacity for assimilation of the ammonium at high levels.

In line with this, no up-regulation of Gln-1;1 in shoots to restore plant growth under high ammonium conditions was observed (Figs. 1A and 3A).

It has been argued that Gln-1;1 may constitute part of a signaling pathway mediating modifications to the root system in response to external N supply (Guan et al., 2014). This argument is supported by the observed increase in the Gln-1;1 expression in roots by high ammonium supply (Figs. 1B; Supplemental Fig. S1A) and significant decrease in root fresh weight in the two Gln-1;1 knock-out mutants grown under high ammonium conditions (Fig. 2C). That Gln-1;1 indeed may interact with Gln-1;2 in alleviating ammonium toxicity is also corroborated by the fact that the double mutant gln1;1:gln1;2 displayed a more severe phenotype than the single gln1;2 mutant under high ammonium treatment. Thus, the gln1;1:gln1;2 double mutant had lower total GS activity (Fig. 4, A and B), lower abundance of total GS1 protein (Fig. 7A), reduced biomass production (Fig. 2, A–C), lower content of Gln (Fig. 4D), and accumulated more ammonium (Fig. 2, D and E) than the single gln1;2 mutant.

Gln-1;3 was also expressed at a relatively high level in shoots (Fig. 1A) but was not up-regulated to relieve...
ammonium toxicity when both of the essential GS1 isogenes, *Gln-1;1* and *Gln-1;2*, were lacking (Fig. 3C). The expression of *Gln-1;4* and *Gln-1;5* was in all cases so low that the PCR products came up as late as the negative controls (data not shown). *Gln-1;5* has previously been reported to encode pollen-specific GS1 (Schmid et al., 2005).

Promoter-GFP expression studies showed that *Gln-1;2* was expressed in mesophyll and vascular cells of the developed leaves (Fig. 8, A–D) as well as in trichomes of the new leaves (Fig. 8E; see also Dubois et al., 1996). This localization indicates that *Gln-1;2* plays a role in export of N from developed source leaves to developing sink leaves. After leaf development, ammonium liberated from protein turnover and amino acid catabolism is assimilated into Gln, which is translocated in the vascular bundles to new sinks (Zhang et al., 2010; Pratelli et al., 2012). Among the new sinks are trichomes that are the first cells forming during new leaf development (Zhou et al., 2013). Our amino acid results showed a significant decrease in Gln content in the *Gln-1;2* knock-out mutants (Fig. 4D), supporting the essential role of *Gln-1;2* in ammonium assimilation.

Responses of GS Protein and Activity to High Ammonium Levels

Regulation of GS may occur at levels of transcription, translation, subcellular localization, subunit assembly, and post-translational modification of the protein and its turnover (Thomsen et al., 2014; He et al., 2015; Seabra and Carvalho, 2015; Wang et al., 2015). While the regulation of GS gene expression has been studied in many cases, much less is known about responses at protein level. Knock-out of *Gln-1;2* resulted in an apparent decrease in GS1 protein content and total GS activity in Arabidopsis shoots (Figs. 4A and 7A). To confirm the essential role played by *Gln-1;2* in contributing to shoot GS1 activity, we used HPLC-based ion-exchange chromatography to separate GS1 and GS2 activities in shoot tissues before activity measurements (Figs. 5 and 6). Three peaks of GS activity were detected, of which the first two contained GS2 and the third GS1 as confirmed by the western-blot analysis (Fig. 5A). The two separate peaks for GS2 were shown to contain different enzymatically active oligomers (Fig. 5B), corresponding to tetrameric and octameric forms. In sugar beet, both of these GS2 oligomers have been shown to be active (Mäck and Tischner, 1990). We optimized the ion-exchange method for separation of GS1 and GS2 activities by using two different linear elution gradients for GS1 and GS2, and by applying each gradient at a slow flow rate. This allowed complete separation of GS1 and GS2, as well as resolution of different active GS2 oligomers. Hence, the set-up circumvented the problems with peak overlap between GS1 and GS2 that have been previously reported (Lothier et al., 2011). The fact that GS2 eluted before GS1, as also previously observed in Arabidopsis (Lothier et al., 2011), *Trientalis europaea* (Parry et al., 2000) and *Pinus sylvestris* (Elmlinger and Mohr, 1992), reflects differences in amino acid sequence and pI of the proteins. The GS2 protein can carry a chloroplast transient peptide that may be removed from the mature protein (Grabowska et al., 2012), leading to a reduction of the pI and the elution time relative to GS1. This presumption with respect to GS2 protein processing can also affect the elution of GS2 itself, resulting in the observed two separate peaks for GS2.

High ammonium supply indeed resulted in increased level of GS1 protein in shoots of both wild-type and knock-out lines (Fig. 7A). In the *gln1;1* mutant, the increase in GS1 protein may reflect a compensatory
rescue derived via up-regulation of Gln-1;2 expression (Fig. 3B). However, the increased abundance of GS1 protein in the gln1;2 mutant and in the gln1;1:gln1;2 double mutant despite lack of up-regulation of the expression of other GSI isoforms (Fig. 3C) suggests post-transcriptional regulation of GSI transcripts in plants exposed to high ammonium. The 5’-UTR of Gln-1;3 may function as a translational enhancer (Ortega et al., 2012) and may have contributed to the increase in GS1 protein in the gln1;2 and gln1;1:gln1;2 knock-out lines under the high ammonium conditions. Post-transcriptional and post-translational regulation may have been involved in maintaining the level of GS2 protein (Fig. 7B) and GS activity (Fig. 4A) in the gln1;1: gln1;2 double mutant in response to high ammonium supply despite a markedly depressed Gln-2 expression (P ≤ 0.01; Fig. 3D). Post-translational regulation by a 14-3-3 protein has previously been reported to play a positive role in GS activation (Finnemann and Schjoerring, 2000; Lima et al., 2006).

The fact that high ammonium treatment caused elevated shoot contents of ammonium in wild-type, gln1;1, and gln1;2 single knock-out mutant plants (Fig. 2D) indicates that the load of ammonium exceeded the capacity for assimilation. This may reflect limitations in the availability of carbon skeletons for ammonium assimilation (Ariz et al., 2011; Vega-Mas et al., 2015). Under such substrate-limiting conditions, the level of GS1 protein would be less critical. The enzyme Glu dehydrogenase (GDH) is known to catalyze deamination of Glu to provide 2-oxoglutarate under conditions with carbon limitation (Fontaine et al., 2012). However, because this process also produces ammonium it would not be useful for alleviation of ammonium toxicity. It is not clear if GDH via its aminating reaction may constitute an additional nitrogen assimilatory pathway (Saraska et al., 2016). This pathway would obviously still depend on the availability of 2-oxoglutarate and would not be feasible under conditions with carbon limitation. Increasing aminating activity of GDH in roots was therefore not likely to be the reason that root ammonium levels in the gln1;2 mutant under high ammonium supply did not increase above those in standard N nutrition (Fig. 2E). That GDH did not play an important role in ammonium detoxification is also suggested by the fact that the gln1;1: gln1;2 double mutant had significantly higher tissue content of ammonium in both shoots and roots under high ammonium supply (Fig. 2, D and E). We conclude that cytosolic Gln synthetase Gln-1;2 is the main isozyme contributing to shoot GS1 activity in Arabidopsis and can be up-regulated to relieve ammonium toxicity, thereby sustaining shoot N assimilation and growth.

MATERIALS AND METHODS

Plant Material

Arabidopsis (Arabidopsis thaliana) ecotype Columbia-0 lines with T-DNA insertion in the 5’-untranslated region, 62 nucleotides upstream of the start codon of Gln-1;1 (qht1, SALK_000459), and the third intron, at 599 nucleotides downstream of the ATG of Gln-1;2 (qht1, SALK_145235) were obtained from the European Arabidopsis Stock Centre.

Homozgyous gln1;1 and gln1;2 mutants were identified by PCR on genomic DNA (Ostergaard and Yanofsky, 2004) with the use of a primer annealing to the left border of the T-DNA (LBA1.3 5’-ATTTGGCGATTCCGGAAC-3’) and the following gene-specific primers: for Gln-1;1 (fwd: 5’-TTCAGTGTCTCAC-CACGGAC-3’ and rev: 5’-TCCCCAATTATATTTAGCATCATTACG-3’) and for Gln-1;2 (fwd: 5’-CACAACCAAGGAACCTCAAAG-3’ and rev: 5’-AACG-GAGAATCGAAAAAGAGC-3’). Two PCR reactions were needed for each plant. One reaction included two gene-specific primers (fwd and rev): wild type and the heterozygous lines resulted in bands of 1106 bps for Gln-1;1 or 1208 bps for Gln-1;2, while the homozygous mutants gave no band. Another reaction included one gene-specific primer (rev) and one T-DNA primer (LBA1.3): bands were obtained for the homozygous mutants and the heterozygous lines, while the wild type was blank.

The gln1;1:gln1;2 double mutants were generated by crossing the single mutants. F1 progeny heterozygous for both genes (qht1, Gln-1/Gln1;1, Gln-1;2) was identified by PCR using the primers above and allowed to self-pollinate. The resulting F2 progeny (160 plants) was screened by PCR, and seven homozygous mutants (qht1, gln1;1, gln1;2, gln1;2) were obtained. Seeds from self-pollination of these plants were used in the following experiments.

Growth Conditions

Hydroponics

Arabidopsis seeds were sterilized in 50% (v/v) ethanol for 1 min followed by washing in 50% (v/v) NaClO with 0.05% (v/v) Triton X-100 for 10 min and finally rinsed five times with sterile water. After vernalization for 48 h at 4°C, the seeds were sown directly on the top of rock wool plugs (GRODAN) placed in 1.5-mL Eppendorf tubes from which the bottom was cut off and the lid removed. The tubes were positioned in floating rafts in 10-L tanks (120 plants per tank) with distilled water placed in a controlled growth chamber under the following environmental conditions: 8:16 h photoperiod at 100 μmol m−2 s−1, day/night air temperature of 22°C/20°C, and 75% humidity of the air. After one week, the distilled water was replaced by aerated nutrient solution (0.5 mM KNO3, 0.5 mM KH2PO4, 0.125 mM MgSO4, 0.125 mM CaCl2, and 0.05 mM Na-Fe-EDTA, pH 5.8). Starting week 3, nutrient concentrations were increased to 1 mM KNO3, 1 mM KH2PO4, 1 mM MgSO4, 0.25 mM CaCl2, and 0.1 mM Na-Fe-EDTA, pH 5.8. From week 4 and onwards, the nutrient source was changed to 2 mM NH4NO3, while the level of other nutrients remained unchanged. During the first three weeks, the nutrient solution was changed once per week thereafter every 2 to 3 d. During the cultivation period, the pH in the nutrient solution around the roots never dropped below 5.6.

Ammonium Treatment

After six weeks with standard N supply (2 mM NH4NO3), plants in the high ammonium treatment were grown at 10 mM (NH4)2SO4, 2.5 mM K2SO4, 1 mM KH2PO4, 1 mM MgSO4, 0.25 mM CaCl2, and 0.1 mM Na-Fe-EDTA, pH 5.8. Plants were harvested 10 d after initiating the high ammonium treatment. Control plants were kept at standard N supply but were supplied with extra potassium (2.5 mM K2SO4, 2 mM NH4NO3, 1 mM KH2PO4, 1 mM MgSO4, 0.25 mM CaCl2, and 0.1 mM Na-Fe-EDTA, pH 5.8) for 10 d. The supplement of K2SO4 was given to avoid disruption of potassium homeostasis in the ammonium-treated plants (Li et al., 2012). At harvest, 24 to 36 plants were collected for each line to produce six replicates. For gene expression and biochemical analysis, materials were immediately frozen in liquid nitrogen and stored at −80°C.

Tissue Ammonium Content

Tissue ammonium was determined after extraction of 50 mg of fresh material in 500 μl of 10 mM ice-cold formic acid (Husted et al., 2000). The aqueous NH4+ containing solution was injected into a NaOH stream and the produced gaseous NH3 measured spectrophotometrically based on the color shift of an indicator solution (catalog no. 50000295, FLAstar 5000 System, FOSS). Ammonium chloride was used to prepare calibration standards.

Amino Acid Analysis

Free amino acids were determined after extraction from 50-mg fresh shoot material in 500 μl of 10 mM ice-cold formic acid with 0.25 mM AABA
(α-aminobutyric acid). After reaction with the AccQ-Taq Ultra Reagent (Waters) in accordance with the manufacturer’s recommendations, derivatized amino acids were separated on an Acquity UPLC system (Waters). Data were analyzed according to the manufacturer’s instructions, using AABA as an internal standard.

Gln Synthetase Activity Assays
Fifty-mg Arabidopsis shoot or root tissue was ground in liquid nitrogen and homogenized in a total volume of 200 μL (50 mM Tris, 2 mM EDTA, 10% [v/v] glycerol, and 10 mM 2-mercaptoethanol, pH 8.0). The homogenates were centrifuged at 16,099g for 3 min at 4°C and the supernatant was analyzed for GS activity.

Total GS activity was assayed by the synthetase reaction (Liu et al., 2010). The reaction buffer contained 70 mM MOPS, 100 mM Glu, 50 mM MgSO4, 15 mM NH4OH, and 15 mM ATP, pH 6.8. Blank controls were obtained by omitting addition of ATP to the reaction buffer. Fifty μL of the samples was incubated with 100 μL of reaction buffer at 37°C for 30 min. The reaction was terminated by adding an acidic FeCl3 solution, consisting of 88 mM FeCl3, 670 mM HCl, and 200 mM trichloroacetic acid. The product, GHA (γ-glutamyl hydroxamate), from a biosynthetic reaction of Glu and hydroxylamine (NH2OH), was quantified spectrophotometrically at 498 nm. Synthetic GHA was used to prepare calibration standards.

Specific shoot GS1 and GS2 activities were measured by the transferase reaction after their separation by HPLC. The transferase assay measures the ability of GS to replace the γ-amino group of Gln with hydroxylamine in the presence of ADP and Na-arsenate (Seiler et al., 1990). The entire HPLC system (model no. 1100; Agilent) was kept in a cold room (4°C) and fractions were collected after column separation and assayed for GS enzyme activity. The supernatant containing crude protein extract was filtered (0.2 μm) using a syringe and 100 μL was injected onto a Mono Q anion-exchange column (5/50 GL, GE Healthcare) connected to the HPLC system. The Mono Q anion-exchange column had been pre-equilibrated with equilibration buffer (25 mM Tris-HCl, 10 mM MgCl2, 1 mM DTT, 10% [v/v] glycerol, pH17.0) before loading. Proteins were separated on the column using two linear gradients from 0.168 to 0.246 M NaCl and 0.246 to 0.39 M NaCl at a flow rate of 0.3 mL min−1. Twenty fractions of 300 μL were collected per analytical run and assayed for GS activity. Collected fractions were mixed with 100 μL buffer (80 mM Tris-HCl, 64 mM Gln, 2.24 mM MnCl2, 25 mM Na-arsenate dibasic, 16 mM NH4OH, and 0.24 mM ADP, pH 6.4) and incubated at 30°C for 30 min. Blanks were obtained by leaving out ADP and Na-arsenate in the reaction buffer. Reactions were terminated by adding the acidic FeCl3 solution and the product GHA was quantified spectrophotometrically at 498 nm. Synthetic GHA was used to prepare calibration standards.

SDS-PAGE and Blue Native-PAGE
Each HPLC-separated protein fraction was concentrated with 10 K centrifugal filters (Amicon Ultra) according to the manufacturer’s instructions. One-half of the concentrated proteins were separated under denaturing conditions using PAGE (SDS-PAGE; 12% Bis-Tris, Criterion XT, Bio-Rad). The SDS-PAGE was run in 1× MOPS (Bio-Rad) at 200 V for 2 h. The other half were separated under non-denaturing conditions using NativePAGE (4–16% NativePAGE Novex Bis-Tris Mini Gels; Life Technologies). Anode running buffer was made from 1× NativePAGE running buffer (Life Technologies), and light-blue cathode running buffer was prepared according to the manufacturer’s instructions. The Blue Native-PAGE was running in a cold room (4°C) at 150 V for 1 h, then with an increased voltage to 250 V for 1.5 h.

Western-Blot Analysis
After PAGE, denatured proteins were electrophoretically transferred to a 0.2-μm pore-size nitrocellulose membrane (Bio-Rad) in 1× transfer buffer (25 mM Tris-base and 192 mM Gly, pH 8.3) at 100 V for 30 min. Native proteins were electrophoretically transferred to 0.2-μm pore-size PVDF membranes (Bio-Rad) in 1× NuPAGE transfer buffer (Life Technologies) at 35 V for 1 h. The membranes were blocked with PBST (8 mM KH2PO4, 3.9 mM KH2PO4, 150 mM NaCl, and 0.05% [v/v] Tween 20, pH 7.2) containing 5% skimmed milk. The blocked membranes were then incubated with 20 μg of anti-GS serum (rabbit IgG) in 20 mL of PBST at 4°C overnight. The antibody was used as a GS-specific antibody raised against the root nodule GS1 of Phaseolus vulgaris (Cullimore and Miflin, 1984). This antibody cross-reacts with all higher plant cytosolic and plastidic GS enzymes so far tested. After several washes with PBST, the membranes were incubated at room temperature for 2 h with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000; Thermo Scientific). The immune complexes were detected using chemiluminescence reagents (Super Signal; Thermo Scientific).

Laser-Ablation Inductively Coupled Plasma Mass Spectrometry
Quantitative protein analysis of GS1 and GS2 was conducted using laser-ablation inductively coupled plasma mass spectrometry to detect signals from the western blot membranes after incubation with primary antibodies (rabbit IgG) against the β-subunit of ATP synthetase (CF1) at 1:2,500 and GS, as described above. Secondary immunoglobulin-conjugated goat anti-rabbit antibodies (1:2,500) facilitated laser-ablation inductively coupled plasma mass spectrometry analysis as described by de Bang et al. (2013).

RNA Extraction and Gene Expression Analysis
Total RNA was extracted from 50 to 100 mg of shoot tissue using a solution of 35% (v/v) phenol, 1% guanidine thiocyanate, 1% ammonium thiocyanate, 0.1 M sodium acetate, and 5% (v/v) glycerol. Proteins were removed by chloroform and RNA was purified by isopropanol and washed with ethanol before resuspension in RNase free water. After DNA digestion with TURBO DNase (Applied Biosystems/Ambion) and the confirmation of RNA quality by gel electrophoresis, reverse transcription was performed using M-MuLV Reverse Transcriptase (New England Biolabs). Template cDNA was synthesized from 2 μg of RNA estimated from the concentration measured by a NanoDrop 2000 spectrophotometer (Thermo Scientific). The resulting cDNAs were further diluted with sterile water for quantitative RT-PCR (RT-qPCR), which was performed with DyNAamo Flash SYBR Green qPCR kit (Finzymes) according to the manufacturer’s instructions, and amplification was measured on a Mx3000PTM (Stratagene). Three replicates were run for each sample using a gene with unknown function Ref. 1 AT4G266410; Czechowski et al., 2005) and Ubiquitin2 (UBQ2; AT2G6170), respectively, as reference genes. Very similar results were obtained (data not shown). The expression data presented in Figures 1 and 3 and Supplemental Figure S1 are based on Ref. 1, which was expressed at approximately the same level as Gln-1-1, while UBQ2 was much more highly expressed. The gene expression analysis showed the fold change in gene expression relative to that of the wild type under standard N (2 mM NH4NO3), determined from the CT values according to Pfaffl (2001). Gene-specific primers for RT-qPCR can be found in Supplemental Table S1.

Cloning of Gln-1;2 Promoter-NLS-GFP-GUS/pCAMBIA 3300 DNA Construct
To analyze the expression pattern of Gln-1;2, the DNA sequence upstream of the gene (promoter sequence) was cloned into a vector from which expression of GFP was driven by the Gln-1;2 promoter. A 1,526-bps DNA fragment containing the upstream region of Gln-1;2 was commercially synthesized with restriction sites at 5′ and 3′ ends: GAGCTC and CCCGGG, for SacI and XmaI, respectively. The DNA fragment was cut with SacI and XmaI (New England Biolabs), and ligated with the plant expression vector pCambia 3300 NLS-GFP-GUS (Nour-Eldin et al., 2006). PCR amplification of the DNA fragment with the vector primers Fwd: 5′-GAAACAGCTATGACATGATTACGAA-3′ and Rev: 5′-AGCAGTCACACAGCATAGTG-3′ followed by DNA sequencing confirmed the insertion of the Gln-1;2 promoter.

Plant Transformation
Wild-type Arabidopsis Columbia-0 plants were transformed with the Gln-1;2 promoter-NLS-GFP-GUS/pCambia 3300 construct using the floral dip method (Clough and Bent 1998). Hundreds of seeds from the transformed plants were sown in soil and transformants were selected by spraying with BASTA. Seeds from self-pollination of the transformants were collected for a further selection of homozygotes.

Detection of the Cell-Type Specific Expression of Gln-1;2
Observations of green-fluorescent nuclei (Chytlova et al., 1999) from Gln-1;2 expression were recorded by a model SP5 confocal microscope (Leica Microsystems). Leaves were cut and mounted in water for microscopic observation. A
The importance of cytosolic glutamine synthetase in nitrogen assimilation and recycling. New Phytol 182: 608–620

The following supplemental materials are available.

**Supplemental Figure S1.** Expression of Gln-1,1, Gln-1,2, and Gln-1,3 in the roots of the mutant and wild-type plants growing under standard N (gray bars) and high ammonium (black bars) conditions.

**Supplemental Table S1.** Gene-specific primers for RT-qPCR.


