

RESEARCH PAPER

The cytosolic glutamine synthetase GLN1;2 plays a role in the control of plant growth and ammonium homeostasis in *Arabidopsis* rosettes when nitrate supply is not limiting

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Abstract

Glutamine synthetase (EC 6.3.1.2) is a key enzyme of ammonium assimilation and recycling in plants where it catalyses the synthesis of glutamine from ammonium and glutamate. In *Arabidopsis*, five *GLN1* genes encode GS1 isoforms. *GLN1;2* is the most highly expressed in leaves and is over-expressed in roots by ammonium supply and in rosettes by ample nitrate supply compared with limiting nitrate supply. It is shown here that the *GLN1;2* promoter is mainly active in the minor veins of leaves and flowers and, to a lower extent, in the parenchyma of mature leaves. Cytoimmunochemistry reveals that the *GLN1;2* protein is present in the companion cells. The role of *GLN1;2* was determined by examining the physiology of *gln1;2* knockout mutants. Mutants displayed lower glutamine synthetase activity, higher ammonium concentration, and reduced rosette biomass compared with the wild type (WT) under ample nitrate supply only. No difference between mutant and WT can be detected under limiting nitrate conditions. Despite total amino acid concentration was increased in the old leaves of mutants at high nitrate, no significant difference in nitrogen remobilization can be detected using ¹⁵N tracing. Growing plants *in vitro* with ammonium or nitrate as the sole nitrogen source allowed us to confirm that *GLN1;2* is induced by ammonium in roots and to observe that *gln1;2* mutants displayed, under such conditions, longer root hair and smaller rosette phenotypes in ammonium. Altogether the results suggest that *GLN1;2* is essential for nitrogen assimilation under ample nitrate supply and for ammonium detoxification.

Key words: Ammonium, *Arabidopsis thaliana*, companion cells, glutamine synthetase, nitrogen assimilation, nitrogen remobilization.

Introduction

In plants, glutamine synthetase (GS, EC 6.3.1.2) is a key enzyme of primary ammonium assimilation, which catalyses an ATP-dependent conversion of glutamate to glutamine using ammonium derived from fertilizer, nitrate reduction, photorespiration, and numerous other sources including the catabolic release of ammonium during senescence. The existence of two different glutamine synthetase isoenzymes,

located in the chloroplast (GS2) or the cytosol (GS1), suggests that glutamine biosynthesis is separated into two independent pathways. In young leaves, glutamine is believed to be mainly formed within the chloroplast through the GS2/glutamate synthase (GOGAT) cycle from the assimilation of NH₄⁺ provided by nitrate reduction and photorespiration (Wallsgrave *et al.*, 1987; Leegood *et al.*,

1995). As leaves senesce and chloroplasts are degraded, chloroplastic enzymes and redox potentials are modified. As a result, the contribution of the GS2/GOGAT cycle decreases (Masclaux *et al.*, 2000). In old senescing leaves, it is proposed that glutamine is synthesized by newly expressed GS1 isoforms (Bernhard and Matile, 1994; Masclaux *et al.*, 2000; Cabello *et al.*, 2006; Martin *et al.*, 2006). Proteolysis of chloroplast proteins would release glutamate that serves as a substrate of the glutamate dehydrogenase (GDH, EC 1.4.1.2), (Purnell and Botella, 2007). GDH would, in turn, provide 2-oxoglutarate to support respiration and NH_4^+ reassimilated by GS1 for export and remobilization (Masclaux-Daubresse *et al.*, 2006). However, the role of GS1 in the ammonium assimilation and nitrogen (N) remobilization process actually appears more complex because several GS1 isoforms encoded by multigene families are found in all plant species. The role of GS1 in nitrogen management, growth rate, yield, and grain-filling has been suggested by the finding of co-localizations between QTLs (quantitative trait loci) for agronomic traits and GS activity (Hirel *et al.*, 2001; Obara *et al.*, 2004). Over-expression of cytosolic glutamine synthetase has also been performed in various plant species (Fuentes *et al.*, 2001; Habash *et al.*, 2001; Oliveira *et al.*, 2002; Jing *et al.*, 2004). Although effects were variable depending on plant species and promoter combinations, improved growth was monitored in many cases. So far, findings for precise physiological function of each GS1 isoform have been limited and those of OsGS1;1 in rice, GLN1-3 and GLN1-4 in maize, and GLN1-5 in tobacco have only been reported using GS1-knockout or GS1-knock-down plants (Brugière *et al.*, 1999; Tabuchi *et al.*, 2005; Martin *et al.*, 2006).

In rice, three *GS1* genes have been identified, while maize and *Arabidopsis* contain five genes. Among the five *GS1* genes (*Gln1-1* to *Gln1-5*) in maize (Sakakibara *et al.*, 1992, 1996; Li *et al.*, 1993), only *Gln1-4* was up-regulated during senescence (Martin *et al.*, 2005, 2006). GLN1-4 was therefore proposed to be involved in the reassimilation of ammonium released during leaf protein degradation. The *gln1-3*, *gln1-4*, and double [*gln1-4/gln1-3*] mutants displayed a sharp reduction of kernel yield whereas N (%) kernel was increased (Martin *et al.*, 2005, 2006). The *gln1-3* and [*gln1-4/gln1-3*] mutants accumulated large amounts of amino acids and ammonia in source leaves located below the ear as a consequence of a dysfunction in N export. In rice, mutants lacking *OsGS1;1* were severely impaired in growth rate and grain-filling (Tabuchi *et al.*, 2005). Total free amino acids were reduced in mutant leaf blades due to low glutamine levels. It was concluded that the *OsGS1;1* gene product, which was located in the companion and parenchyma cells of leaf tissues, might be responsible for the generation of glutamine for remobilization via the phloem.

In *Arabidopsis thaliana*, three genes were initially characterized but only one of them (*Atgsr2*) was shown to be senescence-induced (Bernhard and Matile, 1994). Finally, the whole genome sequence allowed the annotation of two additional *GS1* genes. The previously characterized *Atgsr1*, *Atgsr2*, and *Atgskb6* genes were renamed *GLN1;1*, *GLN1;2*,

and *GLN1;3* respectively, and the newly discovered genes were named *GLN1;4* and *GLN1;5*. Transcriptomic analyses showed that *GLN1;1*, *GLN1;2*, and *GLN1;4* are induced during leaf ageing (Guo *et al.*, 2004). Ishiyama *et al.* (2004) showed that *GLN1;2* was the only *GS1* gene significantly up-regulated by ammonia in *Arabidopsis* roots. Promoter::GFP fusions showed that *GLN1;2* expression was localized in the root vasculature, *GLN1;1* in the root surface-layer, *GLN1;3* in the vascular tissues, and *GLN1;4* in the pericycle cells. The purification of recombinant *Arabidopsis* GS1 isoforms using *Escherichia coli* over-expression systems allowed authors to show that GLN1;2 and GLN1;3 have a low affinity for ammonia while GLN1;1 and GLN1;4 were found to be high affinity isoenzymes (Ishiyama *et al.*, 2004).

Since *Arabidopsis* GS1 isoforms are located in different tissues and/or organs, are not regulated in a similar manner, and exhibit different kinetic properties, it can be presumed that they do not carry redundant roles in plant physiology (Ishiyama *et al.*, 2004; Martin *et al.*, 2006). In this study, the focus was on the *GLN1;2* gene, one of the most highly expressed in leaves. The aim of this study was to determine its role in nitrogen assimilation and recycling in rosette leaves. GLN1;2 protein location and *GLN1;2* promoter activity were analysed. Mutants lacking GLN1;2 were characterized and their physiology and metabolism investigated under limiting and non-limiting nitrate conditions. Nitrogen remobilization was monitored using isotope tracing.

Taken together, results show that GLN1;2 is important for ammonium assimilation and for vegetative biomass when nitrate supply is high.

Materials and methods

Plant material

Homozygous FST_120E03 and SALK_102291 mutant plants (derived from the INRA Versailles and Salk, collections respectively) were identified by PCR using 5'-CCGAATTTGCTCTAACCAC-AGAACCC-3' and 5'-GCGGGAGAGGCCAATCCCTACTAA-CAAGCG-3'. Plants were backcrossed with their wild-types and homozygous mutants were selected again after backcrossing by PCR.

Transgenic plants carrying a *GLN1;2* promoter fused to the *uidA* reporter gene (*pGLN1;2::uidA*; see below) were obtained by floral dipping (Clough and Bent, 1998).

Growth conditions

In culture: Seeds were stratified, sown, and cultivated according to Lemaître *et al.* (2008) at two nitrate supplies, 2 mM nitrate and 10 mM nitrate. Plants were harvested 29, 36, 43, 48, 57, 64, and 71 DAS. Entire rosette (R), six first leaves (6FL), and new leaves (NL), consisting of the young leaves that emerged after the 6FL, were harvested according to Diaz *et al.* (2008) in order to compare the effects of plant ageing and leaf senescence. At each harvesting time, four different bulks of 6FL, NL, and R were collected for each genotype between 10.00 h and 11.00 h, and stored at -80 °C until further experiments.

In vitro: Seeds were sterilized using Barychlore in 95% (v/v) ethanol, then stratified for 48 h. On day 0, seeds were distributed onto plates containing nitrate (5 mM KNO_3 , 2.5 mM KH_2PO_4 , 2 mM MgSO_4 , 2 mM CaCl_2 , 1.5 mM NaFe-EDTA , 70 μM H_3BO_3 ,

14 μM MnCl_2 , 0.5 μM CuSO_4 , 0.2 μM Na_2MoO_4 , 10 μM NaCl , 1 μM ZnSO_4 , 0.01 μM CaCl_2 , 1% sucrose, 0.7% agar pH 6.0). Plates were placed quasi-vertically in growth chamber at 22 °C under 16 h light (70 $\mu\text{mol m}^{-2} \text{s}^{-1}$)/8 h dark cycles. After 7 d, seedlings were individually transferred to square plates containing either (i) nitrate medium (see above), (ii) ammonium medium (similar to nitrate medium except that KNO_3 was replaced by 5 mM NH_4Cl and 5 mM KCl), or (iii) no-nitrogen medium (similar to nitrate medium except that KNO_3 was replaced by 5 mM KCl). Plates were placed quasi-vertically in the same growth chamber. Root growth was monitored every 2 d. After 14 d, shoots and roots were harvested and stored at -80 °C for further experiments.

Metabolite extraction and analysis, total nitrogen, and protein content determination

Amino acids, ammonium, carbohydrates, nitrate, and total nitrogen were determined according to Lemaître *et al.* (2008). Amino acids from phloem exudates were also determined. The protein concentration was determined using a commercially available kit (Coomassie Protein assay reagent, Bio-Rad, Hercules, California, USA).

Phloem exudate preparation

Petioles of the largest leaves were cut off and re-cut under water just before rapid immersion in the collection buffer (10 mM HEPES and 10 mM EDTA, pH 7.5). Exudates of three plants (two leaves per plant) were collected for 5 h, mixed, lyophilized, and further dissolved in 2% 5-sulphosalicylic acid buffered at pH 2 before amino acid determination.

Enzymatic assays and ion exchange chromatography

Plant material (2 g) was extracted in 10 ml of GS extraction buffer according to Masclaux *et al.* (2000). After centrifugation (15 000 g, 15 min at 4 °C), the supernatant was filtered (0.2 μm , GelmanSciences filter) and injected onto a MonoQ anion exchange column (S/50 GL, GE Healthcare) attached to a FPLC system (ÄKTApurifier, GE Healthcare). The Mono Q column had been pre-equilibrated with 30 ml of equilibration buffer (25 mM Tris-HCl, 1 mM MgCl_2 , 10 mM 2-mercaptoethanol, 1 mM EDTA, pH 7.6) before loading. FPLC was performed at room temperature. Protein fractions were eluted from the column using a linear gradient from 0.1 to 0.7 M NaCl at a flow rate of 1.0 ml min^{-1} . 50 fractions (500 μl) were collected and assayed for soluble protein concentration and GS activity (Masclaux *et al.*, 2000). Reaction buffer for GS activity contained glutamate (80 mM), MgSO_4 (20 mM), EDTA (4 mM), NH_2OH (6 mM), ATP (8 mM), and Tris-HCl (50 mM) and was buffered at pH 7.6. Blank was obtained replacing ATP by Tris-HCl (pH 7.6) (v/v). Assays were performed on protein fractions after FPLC or on crude extracts after centrifugation, by incubating samples with reaction buffer (v/2v) at 30 °C for 30 min. After 30 min incubation, the reaction was stopped adding a solution containing FeCl_3 , TCA, and HCl to obtain as final concentrations in the reaction mix 185 mM, 100 mM, and 2.75% of these compounds, respectively. After centrifugation the optical density of mixes was measured spectrometrically at 540 nm. After activity measurement, aliquots were denatured for SDS-PAGE and Western blot assays.

Gel electrophoresis and protein gel Western blot analysis

GS1 and GS2 isoforms were separated by SDS-PAGE according to Masclaux *et al.* (2000). Antibodies raised against the synthetic peptide AYGEGERNRLTG were used to detect both GS1 and GS2 isoenzymes (Lemaître *et al.*, 2008).

^{15}N tracing experiments

Two different labelling experiments were performed to determine ^{15}N remobilization (i) from leaf to leaf at the vegetative stage

(Experiment 1), and (ii) from rosettes to seeds (Experiment 2) according to Diaz *et al.* (2008). In Experiment 1, 18 plants were grown in hydroponics with a ^{15}N nutritive solution. At T0 (35 DAS), the roots (R) and first leaves (FL) of six plants were harvested and the ^{15}N nutritive solution was replaced by unlabelled ^{14}N nutritive solution. The 12 remaining plants were used for the chase period. The second harvest was carried out at T1, 42 DAS, when seven new leaves (NL) had emerged. A third harvest was performed at T2, 49 DAS. At T1 and T2, plants were dissected as R, FL, and NL. Experiment 2 was conducted using plants grown on sand under high (10 mM) or low (2 mM) nitrate conditions, according to Lemaître *et al.* (2008). ^{15}N labelling was performed at 40 DAS and 42 DAS. Plants were harvested at the end of their cycle when seeds had matured and the rosette was dry. Samples were separated as (i) dry parts [rosette+stem+cauline leaves+silique] and (ii) total seeds. Four replicates (plants) were harvested for each genotype and labelling experiments were carried out twice. Unlabelled samples were used to determine ^{15}N natural abundance.

Determination of total nitrogen content and ^{15}N abundance and partitioning

^{15}N abundance ($A\%$) and nitrogen concentrations ($N\%$, mg. 100 mg^{-1} dry weight) were determined on dry matter according to Diaz *et al.* (2008). The ^{15}N enrichment ($E\%$) was calculated as ($A_{\text{sample}}\% - A_{\text{control}}\%$). The absolute quantity of ^{15}N contained in the sample x was defined as $Q_x = DW_x (A_x\% - A_{\text{control}}\%) \cdot N\%_x$. The Q value of the whole plant (wp) was measured and $Q_{wp} = \sum Q_x$. The partition $P\%$ of ^{15}N in the organ y was calculated as $P\%_y = [E\%_y \cdot DW_y \cdot N\%_y] / [E\%_{wp} \cdot DW_{wp} \cdot N\%_{wp}] = Q_y / Q_{wp}$.

RNA extraction, reverse transcription (RT), and qPCR analysis

Total RNA isolated using Trizol (Invitrogen, Carlsbad, California, USA) were used for reverse transcription. First strands were synthesized using M-MLV reverse transcriptase and oligo (dT) primers (Invitrogen, Carlsbad, California, USA). The PCR was performed using a Mastercycler® ep realplex instrument (Eppendorf, Hamburg, Germany) and RealMasterMix SYBR Green (5 Prime, Hamburg, Germany) according to the manufacturer. The *EF1 α* gene (At5g60390) or *UBQ2* (At2g36170) was used as a constitutive reference. Gene-specific amplification of each primer couple (see Supplementary Table S1 at JXB online for primer sets) was determined by sequencing the unique product of an RT-PCR.

Immunolocalization using indirect immunofluorescence analysis

Immunolocalization was performed on leaf and stem sections according to Masclaux-Daubresse *et al.* (2006) with the following modifications: (i) slides were incubated with the same rabbit antibodies used for Western blots; (ii) afterwards slides were incubated in Evans Blue solution (0.001% in PBS; 10 min) to decrease dead cell autofluorescence; and (iii) after incubation with the secondary antibodies (goat anti-rabbit IgG labelled with Alexa 488; Molecular Probes) slides were treated with DAPI. Immunofluorescence was observed using a spectral confocal laser-scanning microscope as described by Masclaux-Daubresse *et al.* (2006).

GUS-based construct and GUS staining

Amplifying 2500 bp of the *GLN1;2* promoter was performed on F15E12 BAC matrix using the iProof™ high Fidelity PCR Kit (Bio-Rad, France) and the 5'-AAAAAGCAGGCTATTTTACCAAGAGACCATCCACA-3' and 5'-AGAAAGCTGGGTGGT-TGCAAGAAGAAACAAGAAGA-3' primers. Gateway cloning in the pGWB3 vector was performed according to the manufacturer (Invitrogen, Carlsbad, California, USA). Plants carrying the *pGLN1;2::uidA* construct were stained for 4 h in 50 mM Na_3PO_4 pH 7.0, 5 mM ferricyanide, 5 mM ferrocyanide, 0.05% Triton X-100, and 5-bromo-4-chloro-3-indolyl glucuronide (1 mg ml^{-1}),

then destained in ethanol and observed under light microscope (Axioplan 2, Zeiss, <http://www.zeiss.com/>).

Statistics

The significance of differences between data was determined using the *t* test on XLSTAT2007.

Results

GLN1;2 expression is higher in the rosette leaves and under high nitrate supply compared with low nitrate supply

Glutamine synthetase (GS) and asparagine synthetase (AS) catalyse ammonium assimilation in the roots and leaves of plants. The mRNA contents of AS, GS1, and GS2 coding genes were monitored by real-time PCR according to Ishiyama *et al.* (2004) and using the specific primers listed in Supplementary Table S1 at JXB online. The *GLN1;2* mRNA steady-state levels were monitored in different plant organs of *Arabidopsis* and it was shown that they were higher in rosette leaves than in roots, cauline leaves, and stems (Fig. 1A). This was in good accordance with the fact that among the five *GLN1* genes of *Arabidopsis*, *GLN1;2* is one of the most highly expressed, especially in leaves (see Supplementary Fig. S1 at JXB online). *Arabidopsis* rosettes from plants grown at high (10 mM, HN) or low (2 mM, LN) nitrate supplies for 36 d were harvested and *GLN* and *ASN* mRNA contents were determined (Fig. 1B, C). The *GLN1;2* mRNA level was higher than all the *GLN1* mRNA at HN

but similar to *GLN1;1* and *GLN1;3* at LN. Compared with LN conditions, *GLN2* (encoding GS2), *GLN1;2*, and *ASN2* mRNA levels were significantly increased at HN, also to a lower extent *ASN3* (Fig. 1B, C). By contrast, *GLN1;1* and *GLN1;4* mRNA contents were significantly decreased at HN compared with LN. The positive effect of nitrate on *GLN1;2* expression has also been observed in roots by Gifford *et al.* (2008), (see Supplementary Fig. S2 at JXB online).

pGLN1;2::uidA fusion activity is detected in nutrient-loading veins, old parenchymal cells and trichomes

To obtain information on the expression pattern of *GLN1;2* *in situ*, histochemical staining of GUS activity was performed on three independent transformants carrying the *pGLN1;2::uidA* construct. Similar results were obtained with all of these independent lines. The reporter gene activity was undetectable in root hairs but a weak activity was observed at the base of the main root (not shown). In the rosette, a strong GUS staining was observed in the youngest emerging leaves and in the apical meristem (Fig. 2A). During young leaf development, GUS activity progressively disappeared from the tip to the bottom of the leaf, giving rise to a white-blue pattern as shown in Fig. 2B. At the apical white part of the leaves only tertiary and quaternary veins remained blue (Fig. 2B). In developing leaves, GUS staining was found in trichomes (Fig. 2C). In mature leaves, GUS activity was mainly located in tertiary and quaternary veins (Fig. 2D), while the primary and secondary veins showed no signal. Figure 2E shows mature leaves stained with X-Gluc substrate for a longer time than in Fig. 2D. When mature leaves were ageing, GUS staining was maintained in minor veins and reappeared in leaf blades. GUS staining was expanding with ageing from the tip to the bottom of the leaves, i.e. from the older to younger tissues (Fig. 2A). GUS staining in the vein was confirmed in petals and at the top of flowering stems (Fig. 2F). It can be seen that GUS staining was also in developing seeds (Fig. 2G).

Characterization of two *gln1;2* knockout mutants in *Col-8* and *WS* backgrounds

In order to investigate the role of *GLN1;2* in ammonium assimilation under low and high nitrate supplies, homozygous T-DNA insertion mutants were isolated. The T-DNA insertions FST_120E03 and SALK_102291 are located to the last intron and exon of the *GLN1;2* gene (At1g66200), respectively (Fig. 3A). Homozygous E03 and S602 mutants, carrying FST_120E03 and SALK_102291, respectively, were isolated after backcrossing with WS and Col-8 accessions, respectively, and segregation. No distorted segregation pattern was observed using a PCR assay to detect homozygous and heterozygous lines.

While a large amount of *GLN1;2* mRNA was measured in the rosettes and roots of wild-type plants (Figs 1A, 3B) using quantitative and semi-quantitative RT-PCR (see Supplementary Table S2 at JXB online), no *GLN1;2*

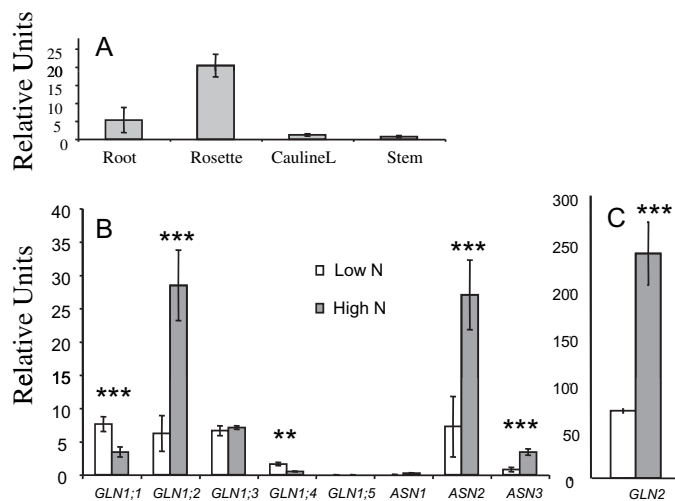


Fig. 1. Expression of *GLN1;2* in *Arabidopsis* organs and under low and high nitrate supplies. (A) Steady-state amounts of *GLN1;2* mRNA in roots, rosettes, cauline leaves (CaulineL), and stems of Col-8. Means and standard deviation are for three biological repetitions of three plants each. Steady-state levels of *GLN1;1*, *GLN1;2*, *GLN1;3*, *GLN1;4*, *GLN1;5*, *ASN1*, *ASN2*, and *ASN3* mRNA (B) and *GLN2* mRNA (C) in 36-d-old rosettes grown at high (10 mM nitrate, High N, grey bars) and low (2 mM nitrate, Low N, white bars) nitrate supplies. Means and standard deviation are for three biological repetitions of three plants each. Significant differences estimated using Student *t* test were indicated by ***P* < 0.01 and ****P* < 0.001.

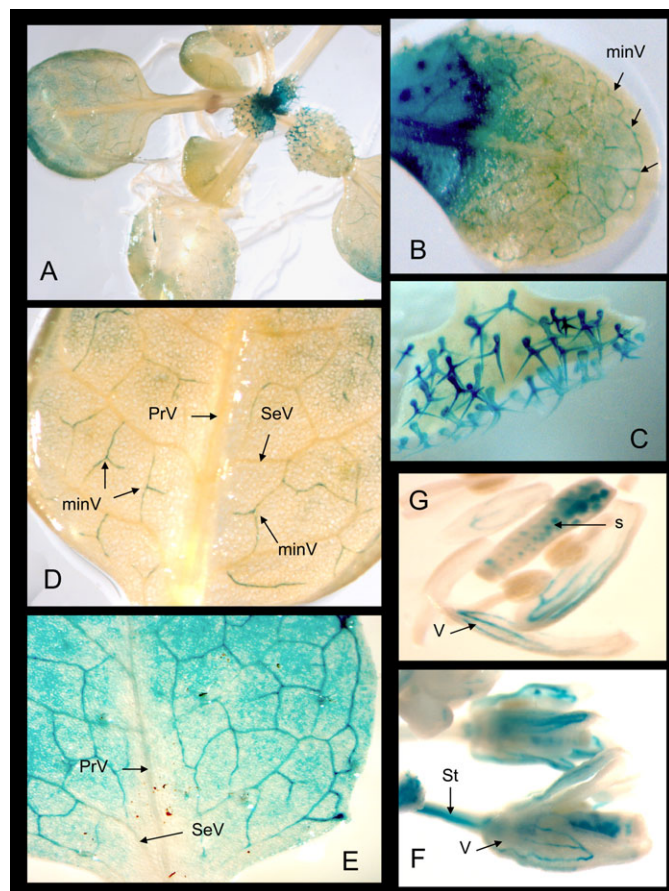


Fig. 2. Expression of transcriptional fusion between *GLN1;2* promoter and *uidA* reporter gene. GUS staining was observed in the whole rosette (A), in the youngest emerging leaves (B; minV: minor vein), in trichomes of young expanding leaves (C), in minor veins of mature leaves (D: PrV, primary vein; SeV, secondary vein; minV, minor vein), in the parenchyma of mature leaves after long GUS staining (E), in young stems (F: St, stem), in petal veins (F, G: V, petal vein) and in seeds (G: s: seeds). Similar staining was observed on three independent *pGLN1;2::uidA* homozygous transformants.

mRNA can be detected in any of the E03 and S602 plant tissues (Fig. 3B; see Supplementary Table S2 at *JXB* online).

GS activities and GS1 and GS2 isoenzyme contents were determined in roots and rosettes following ion-exchange chromatography. Similar results were obtained on WS/ E03 and Col-8/ S602 (Fig. 3C,D; see Supplementary Fig. S3 at *JXB* online); Figure 3C and D show results obtained on Col-8 and S602. In wild-type shoot extracts, two GS activity peaks (I and II, Fig. 3C) were detected after ion-exchange chromatography. In mutant shoots, only peak I can be detected. Western blot analyses of peak I and peak II GS contents showed that wild-type peak I only contained GS2 whereas wild type peak II contained both GS2 and GS1 (Fig. 3C, WT). By contrast, the mutant's fractions corresponding to peak I and II mainly contained GS2 protein (Fig. 3C, Mut). A very weak GS1 signal was, however, detected in the S602 fractions eluting with peak II, presumably corresponding to some GS1 encoded by *GLN1;1*,

GLN1;3 or *GLN1;4*. In wild-type roots a single peak, corresponding to the shoot peak II only contained GS1 proteins (Fig. 3D). Mutant roots showed a very weak peak II (Fig. 3D) compared with the wild-type and GS1 was barely detected on Western blots.

Altogether, the results indicate that S602 and E03 are *gln1;2* knockout lines (Fig. 3; see Supplementary Fig. S3 *JXB* online).

GLN protein is absent from companion cells in *gln1;2* mutants

GLN1;2 localization was estimated by an indirect immunofluorescence method using confocal microscopy by comparing labelling of mutant and wild-type tissues. In both wild-type and mutant leaf sections, labelling was similarly detected within the chloroplasts of epidermal, parenchyma, and mesophyll cells and in the cambium layer. In the cytosol of parenchyma and epidermis cells, labelling was low and difficult to discriminate from the background. For that reason it was not possible to determine clearly whether *GLN1;2* was present in the cytosol of parenchyma cells (see Supplementary Fig. S4 at *JXB* online). The phloem tissues that can be clearly identified on leaf sections were those of primary and secondary veins and no difference could be detected between mutant and wild-type. This can be explained by the fact that the *GLN1;2* promoter is mainly active in tertiary and quaternary veins that were too difficult to identify on our slides.

By contrast, differences between wild-type and mutant were clearly detected on the transverse stem slides. In wild-type stems, fluorochrome labelling was detected within the chloroplasts of cortex and epidermal cells, in the cambium cell layer, in some phloem parenchyma cells containing chloroplasts, and in the phloem companion cells (Fig. 4A–D). Labelling of the wild-type phloem tissue was examined further and a co-localization with DAPI-stained nuclei was observed (Fig. 4D). Co-localizing the fluorochrome specific-labelling and DAPI-stained nuclei indicated that the GS protein was located in small cells containing nuclei that were identified as companion cells. GS labelling was absent from the nucleus-deficient sieve elements. In mutants, fluorochrome labelling was detected in the chloroplasts of cortex and epidermal cells, in the cambium cell layer, in a few phloem parenchyma cells but it was absent from the companion cells (Fig. 4E–G). The observed differences between mutants and wild-types unambiguously showed that *GLN1;2* is located within the companion cells of the phloem.

Mutant growth and flowering are differentially affected by low and high nitrogen growth conditions

Plants were grown at both high (10 mM) and low (2 mM) nitrate levels under short days in order to investigate the growth and developmental differences between mutant and wild-type plants.

No differences were found between mutants and wild types for germination rate and time, and for flowering time at low or high nitrate (not shown). Rosette growth and

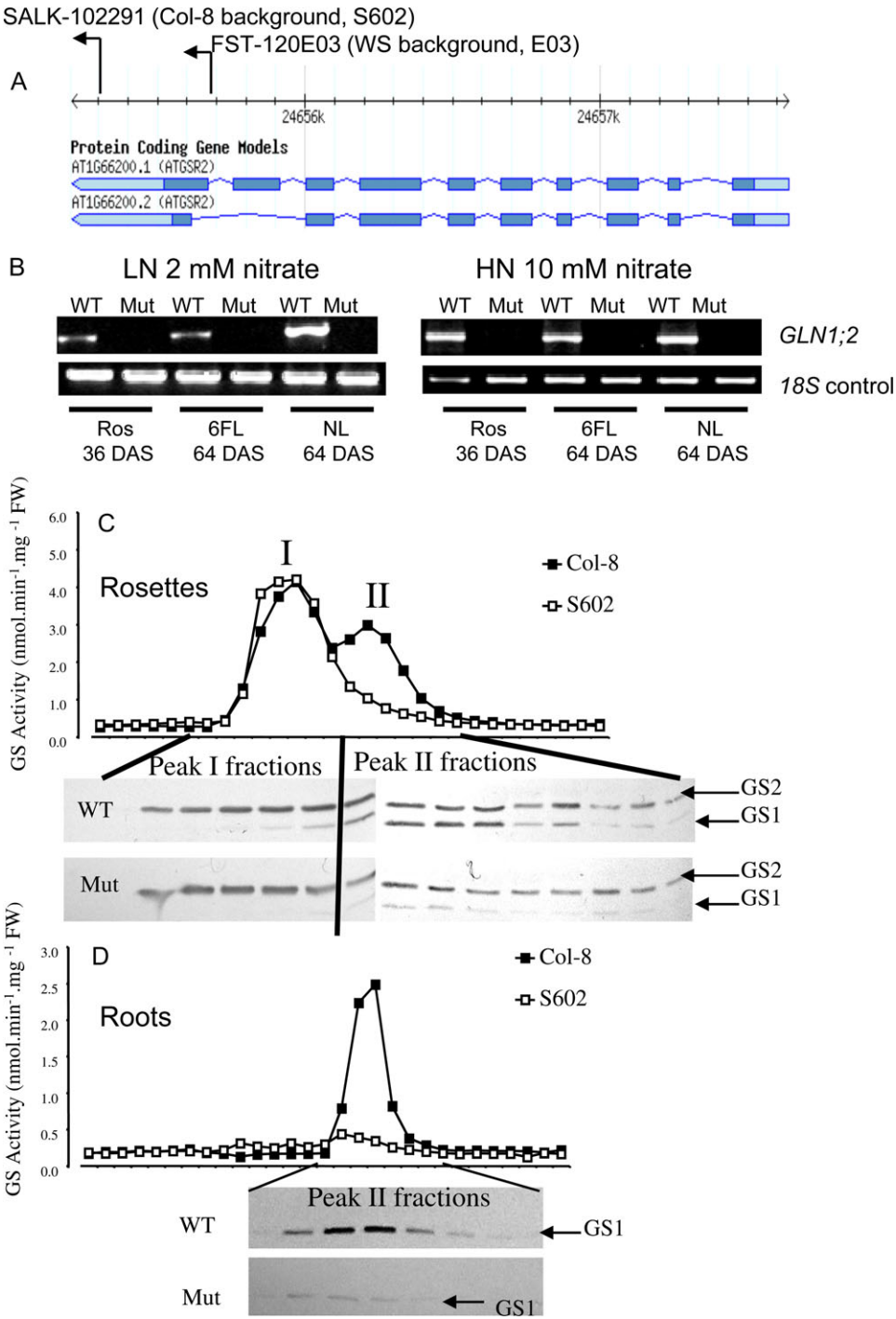


Fig. 3. Characterization of two *gln1;2* knockout mutants in Col-8 and WS backgrounds. (A) T-DNA insertions FST_120E03 and SALK_102291 are located to the last intron and exon of the *GLN1;2* gene (At1g66200), respectively. (B) Comparison of the steady-state levels of *GLN1;2* mRNA in WS wild type (WT) and E03 mutant (Mut) with low nitrate (LN) and high nitrate (HN) supplies, in 36-d-old rosettes (Ros), in the senescing six first leaves of 64-d-old plants (6FL) and new leaves of 64-d-old plants (NL). Similar results were obtained comparing Col-8 and S602 (not shown). DAS, days after sowing. (C, D) Comparison of GS activities and isoenzymes in rosettes (C) and roots (D) of *gln1;2* S602 mutant (Mut) and Col-8 (WT) after ion-exchange chromatography. Two peaks (I and II) of GS activity were analysed by Western blots for GS contents [GS2 (upper band) and GS1 (lower band)]. Similar results were obtained when comparing E03 and WS (see Supplementary Fig. S3 at JXB online).

biomass was not different between mutant and wild type at low nitrate (Figs 5A, 6A). By contrast, at high nitrate supply, rosette surface area and rosette biomass appeared significantly lower in mutants (Figs 5B, C, 6B). Such

biomass phenotype was also observed *in vitro* on plants grown on MS rich medium (Fig. 5D). The difference in rosette biomass was due to leaf growth since mutant and wild-type leaf numbers were the same. There was no

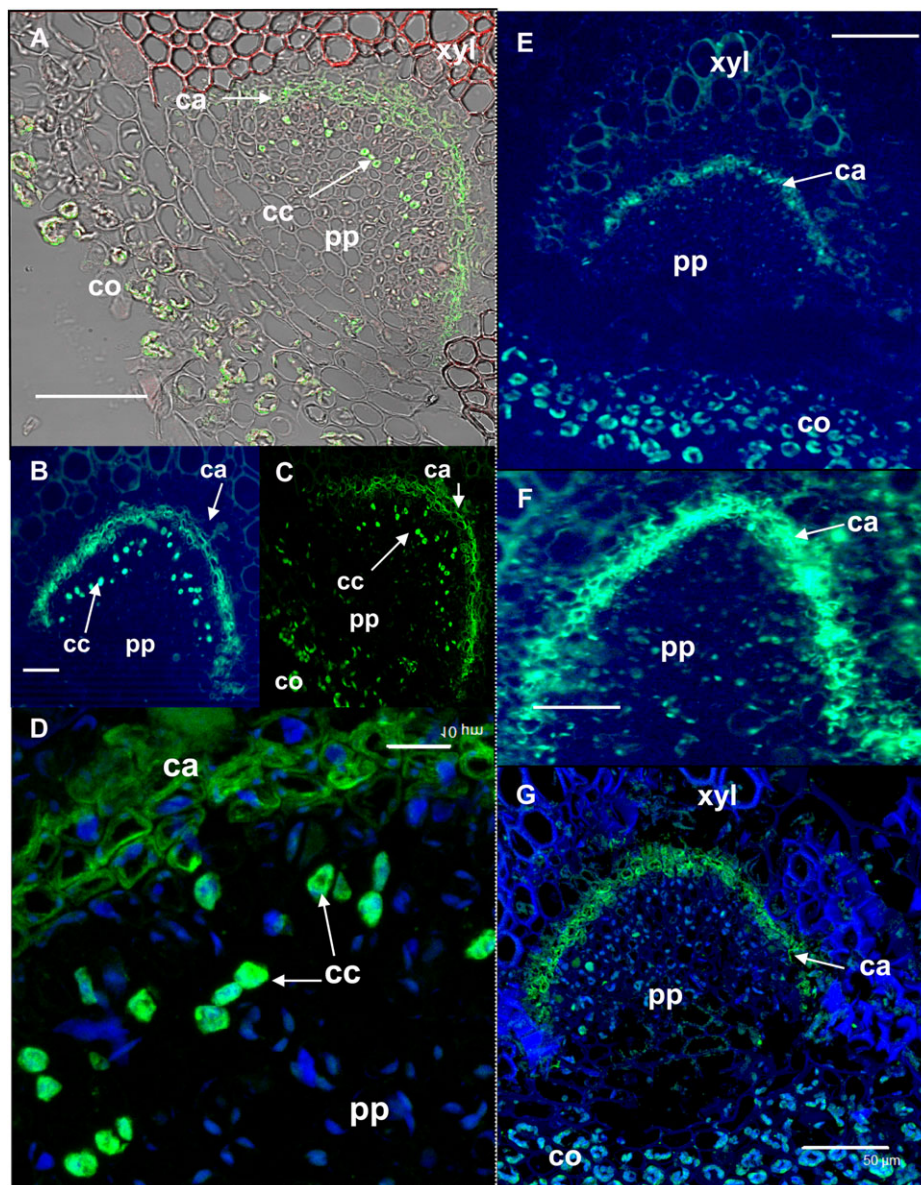


Fig. 4. Immunolocalization of GS using indirect immunofluorescence analysis on Col-8 (A, C), WS (B, D), E03 (E, G), and S602 (F). Immunolocalization was performed with the same GS polyclonal antibodies as for Western blotting. GS antibodies were detected using secondary goat anti-rabbit IgGs labelled with Alexa 488. In wild-type (A, B, C, D), fluorochrome labelled the chloroplasts of phloem parenchyma (pp) cells and cortex (co) cells and the cambium (ca). No labelling was detected in xylem (xyl). In mutants (E, F, G) labelling of chloroplasts in the cortex (co) and some phloem parenchyma cells (pp) and of cambium (ca) was similar, but there was no labelling in the phloem companion cells (cc). DAPI and Alexa 488 overlaps (D, G) show the presence of nuclei within the fluorochrome-labelled companion cells (cc) of wild types (D) and confirm the absence of labelling in the companion cells of mutants (G). White lines indicate scales of 50 μ m (A, B, C, E, F, G) and 10 μ m (D).

significant delay in flowering time at high nitrate, however, the mutant stem growth rate was slower (Fig. 5E).

Total GS activity and GS1 protein content are affected at high nitrate in the gln1;2 mutant but unchanged at low nitrate supply

The effect of the mutation on total GS activity was determined. Protein concentrations in both mutant and wild-type accessions were similar and equal to that published by Lemaître *et al.* (2008). For that reason the GS

activities presented in Fig. 7 were expressed on a protein basis. At high nitrate supply, GS activity in rosettes was decreased in the mutant by more than 50% (Fig. 7A) while at low nitrate, the difference between mutant and wild-type plants was only significant before 43 d after sowing (Fig. 7B). GS protein contents were estimated by Western blotting. At 2 mM nitrate, the GS2 and GS1 protein contents in rosette leaves were similar between wild-type and mutant, except at 36 DAS (Fig. 7C). At 36 DAS, the GS1 content was lower in the mutant compared with the wild type. Under high nitrate conditions, GS1 protein

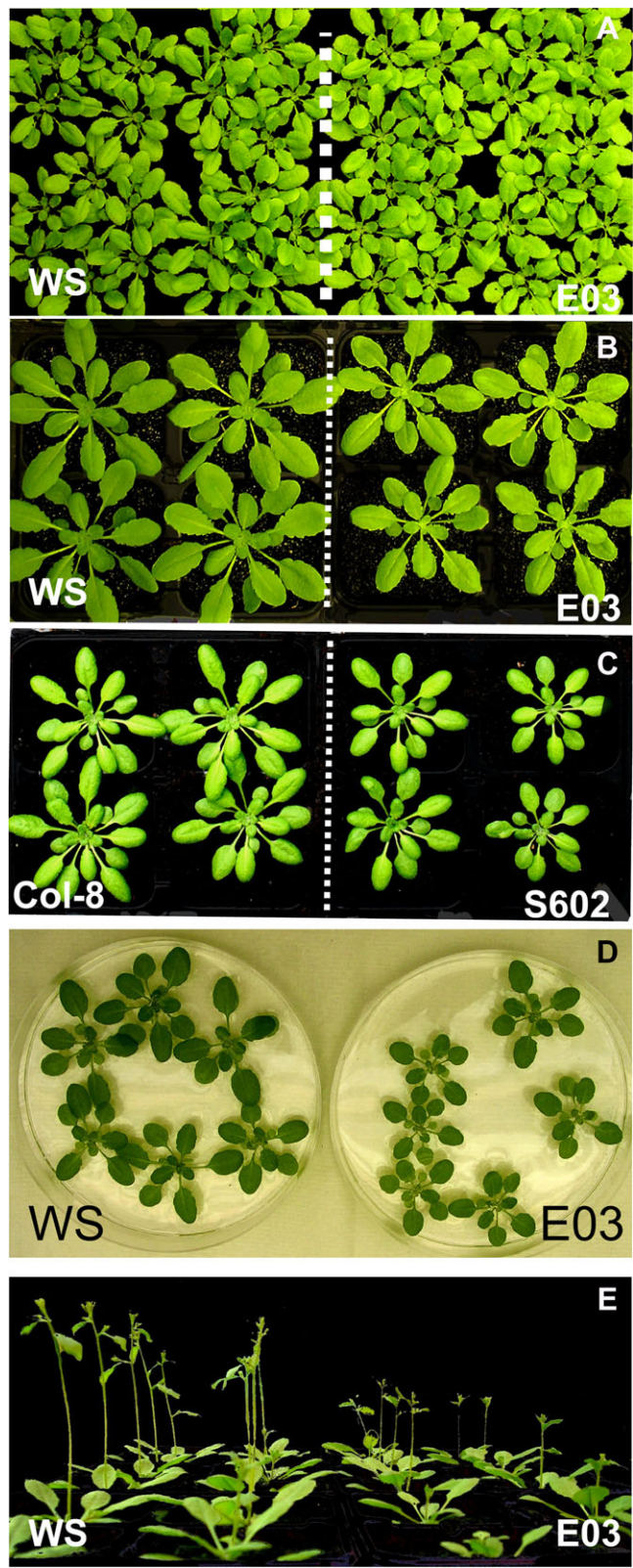


Fig. 5. Plant growth and flowering phenotypes of *gln1;2* knockout mutants (E03 and S602) and wild types (WS and Col-8) when grown in compost under 2 mM (A) or 10 mM (B, C, E) nitrate or on MS/2 1% glucose plates (D).

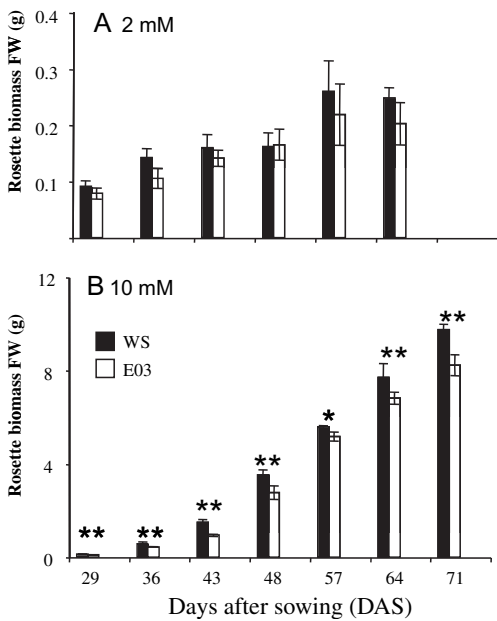


Fig. 6. Comparison of plant biomass. Rosette fresh weight (FW) in WS (black bars) and E03 (white bars) plants grown at low (A) and high (B) nitrate for 71 d. Means and standard deviations are given ($n=4$ plant repeats). Stars indicate significant differences between mutant and wild-type lines (* $P < 0.05$; ** $P < 0.01$). Similar significant differences in rosette biomass were observed for Col-8 and S602 under high nitrate supply but not under low nitrate.

contents were clearly lower in mutant compared with the wild type and at all time points (Fig. 7C). This difference was consistent with the higher expression of *GLN1;2* at high nitrate compared to the other *GLN1* genes and compared to its own expression at low nitrate (Fig. 1).

The gln1;2 mutation affects ammonium levels at high nitrate supply only but poorly modify amino acid contents

Previously, Diaz *et al.* (2008) showed that *GLN1;2* mRNA levels increased during leaf ageing. Therefore, in order to detect potential leaf ageing-related effects due to the mutation, shoot material was separated into batches containing the six first leaves (6FL) and new leaves (NL), as previously described by Diaz *et al.* (2005, 2008). Total nitrogen, nitrate, ammonium, amino acid, and sugar levels were measured in the shoots of plants grown at low and high nitrate. No difference in total nitrogen, nitrate, and sugar concentrations was detected between mutant and the wild type (data not shown).

At low nitrate supply, there was no significant difference in ammonium (Fig. 8A), total amino acids (Fig. 8B), and individual amino acids (see Supplementary Figs S5 and S6 at JXB online) between mutants and their wild-types, except at 29 DAS in 6FL and NL for ammonium (Fig. 8A). Interestingly, 29 DAS is one of the rare time-points where GS activity was lower in the *gln1;2* mutant compared with the wild type under low nitrate supply (Fig. 7B).

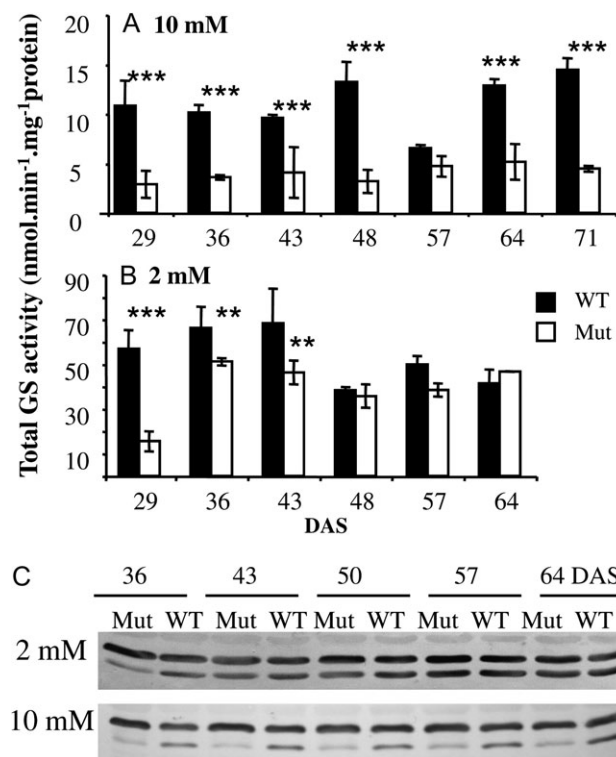


Fig. 7. Comparison of GS activity and GS protein content in WS (black bars, WT) and E03 mutant (white bars, Mut) rosettes. GS activity was determined in rosettes of plants grown at high (A, 10 mM) and low (B, 2 mM) nitrate for 71 d. Means and standard deviations are shown ($n=4$ plant repeats). Significant difference between mutant and the wild type were determined using Student t test: *** $P < 0.001$, ** $P < 0.01$. GS1 and GS2 contents in rosettes of E03 (Mut) and WS (WT) plants grown at low (2 mM) and high (10 mM) nitrate were analysed by Western blots (C). The upper band corresponds to GS2 and the lower band to GS1. Similar results were obtained when comparing the S602 *gln1;2* mutant with the Col-8 wild type.

When grown under high nitrate, the 6FL and NL of mutant plants accumulated ammonium (Fig. 8A). This was consistent with the large GS activity decrease observed in mutants under ample nitrate supply (Fig. 7A). Although the large increase of ammonium in the NL of mutants at high nitrate supply, total amino acid concentrations in the NL of mutants were unchanged compared with wild types (Fig. 8B). By contrast, while ammonium increase was similar in the 6FL and the NL of the mutant at high nitrate supply, total amino acid concentration only increased in the 6FL of mutant compared with the wild type (Fig. 8B). This increase was observed even at an early developmental stage when 6FL were still young green leaves.

Individual amino acid proportions (measured as % of total amino acids) were monitored in the NL and the 6FL of mutants and wild types. No difference was found in individual amino acid proportions in mutants compared with wild types at low nitrate for 6FL and NL and at high nitrate in NL (see Supplementary Figs S5 and S6 at JXB online). At high nitrate, differences were found between the

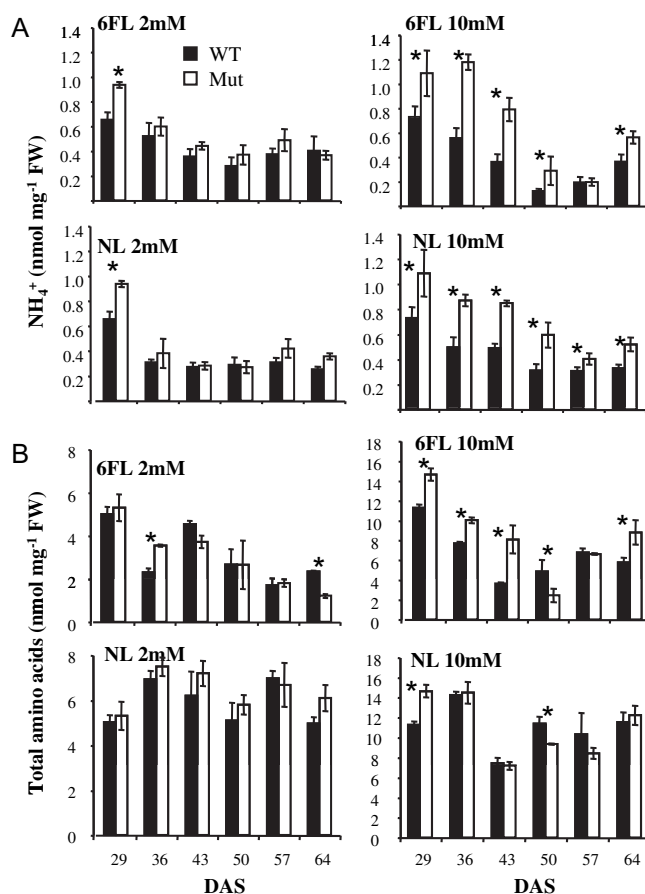


Fig. 8. Ammonium (A) and total amino acid (B) levels in the six first leaves (6FL) and new leaves (NL) of plants grown at low (2 mM) and high (10 mM) nitrate. Ammonium concentrations (A) were higher in the six first leaves (6FL) and the new leaves (NL) of mutant E03 (white bars) when compared with WS (black bars). Free amino acid levels (B) were higher in the 6FL of mutant E03 (white bars) but similar to the wild-type (black bars) in the NL. Similar ammonium and amino acid accumulations were observed in S602 compared with Col-8. Means and standard deviations are given ($n=4$ plant repeats). An asterisk indicates significant difference between mutant and wild-type plants (Student test $P < 0.05$).

6FL of WT and mutants in a reproducible way (see Supplementary Figs S5 and S6 at JXB online). Results of amino acid determination performed on the 6FL of E03 and WS (Fig. 9A) and of Col-8 and S602 (Fig. 9B) at 64 DAS show that the % of individual amino acids were different depending on the Col-8 and WS genetic backgrounds. For example, Col-8 showed higher serine % and threonine % than WS. Despite these differences, similar effects of *gln1;2* mutations were observed in both backgrounds. Higher glutamine %, alanine % and proline % were found in E03 and S602 compared to their wild types. These higher % were compensated in E03 by a lower glutamate % and in S602 by a lower serine %.

The composition of individual amino acids in WT and mutant phloem exudates was also monitored. There was no difference during early plant development, but a significant and large decrease in proline proportion was observed in

the phloem exudates of the old rosette leaves of E03 mutant at 64 DAS (see [Supplementary Fig. S7](#) at *JXB* online). Such a difference was not found to be significant for the other mutant S602 and it was difficult to repeat this result since harvesting exudates and amino acid determination in exudates was difficult, depending of exudate quality, thus explaining the variations in our results.

Nitrogen remobilization estimated using global ¹⁵N labelling is not affected by the gln1;2 mutation

In order to investigate whether the accumulation of ammonium and amino acids in the 6FL of mutants was related to an altered nitrogen remobilization, the nitrogen remobilization efficiency from leaf to leaf and from rosettes to seeds was monitored by ¹⁵N tracing according to [Diaz et al. \(2008\)](#).

The first experiment was performed at the vegetative stage to determine the amount of ¹⁵N remobilized from roots and old leaves to the new leaves. Plants were grown in a ¹⁵N- containing hydroponic solution for 35 d to label the source organs used for N-remobilization. The label was then chased by replacing ¹⁵N by ¹⁴N in the nutritive solution. Two harvest time points were chosen during the chase period (T1=42 DAS and T2=49 DAS). The amount of ¹⁵N contained in the leaves that emerged during the chase period was monitored and considered as the quantity of remobilized nitrogen. Since ¹⁵N amounts depended on the former ¹⁵N enrichment in the source organs and on the respective size of sink and sources, partitioning of ¹⁵N was considered. [Table 1](#) represents the ¹⁵N partitioning (P%¹⁵N) and the biomass partitioning (P%DW) in roots, first leaves, and new leaves. This shows that there were no significant differences of P%DW and P%¹⁵N between mutant and wild-type plants at T1 and T2 ([Table 1](#)).

The second experiment was performed in order to measure ¹⁵N remobilization to the seeds. Nitrogen labelling was performed early enough during plant development to be sure that all of the nitrogen provided to the plants had been fully assimilated into amino acids and proteins ([Diaz et al., 2008](#); [Lemaître et al., 2008](#)). Results showed that mutant and wild-type yields, harvest index (HI ie. P%DM of seeds), N and C concentration in seeds and dry remains, and the C/N ratio in seeds were not different between genotypes at both low and high nitrate conditions ([Table 2](#)).

Comparison of P%DW and P%¹⁵N between mutant and wild-type lines performed as vegetative and reproductive stages showed that the *gln1;2* mutation did not affect N-remobilization from leaf to leaf nor from rosettes to seeds.

The gln1;2 mutants exhibited reduced biomass and hairy roots when grown in vitro with NH₄⁺ as the sole nitrogen source

In order to investigate whether *gln1;2* mutants are differentially tolerant to ammonium *GLN1;2* mRNA contents were first examined in roots and shoots of *in vitro* plants transferred or not to ammonium-containing medium on vertical plates for 7 d. The results confirmed those of [Ishiyama et al.](#)

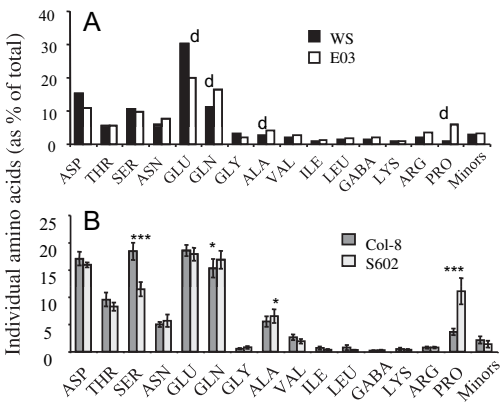


Fig. 9. Individual amino acid proportions are changed during ageing in the six first leaves of *gln1;2* mutants at high nitrate supply. Individual amino acid (as % of total) in the 6FL of E03 (white bars) and WS (black bars) plants grown at high nitrate were determined at 64 DAS (A) on a mix (equal total amino acid amount) of four individual plant repeats (no SD). Other time points are presented in [Supplementary Fig. S6](#) at *JXB* online and the d letter indicates differences that were observed at all the time points. Individual amino acid % in the 6FL of Col-8 (dark grey bars) and S602 (light grey bars) plants grown at high nitrate were determined at 64 DAS (B) on four individual plant repeats. Mean and SD are shown and significant differences between Col-8 and S602 are indicated by **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

Table 1. Comparison of the partitioning of dry weight (P%DW) and total ¹⁵N (P%¹⁵N) of the roots, first leaves (labelled during the pulse period) and the new leaves (appeared during the chase period)

The partitioning P% (in % of the whole plant) of the DW is the ratio [(organ DW in mg).(whole plant DW in mg)⁻¹]. The partitioning P% (in % of the whole plant) of ¹⁵N is the ratio [(μg ¹⁵N in the organ)/(μg ¹⁵N in the whole plant)⁻¹]. P% has been calculated from six individual plant repeats. Means and SD are presented. Three harvest times have been done. T0 is the end of the pulse chase.

Harvest time	Genotypes	Roots		First leaves		New leaves	
		Mean	SD	Mean	SD	Mean	SD
P%DW	T0 (35 DAS)	Col	27%	±9%	73%	±9%	
		S602	23%	±9%	77%	±9%	
	T1 (42 DAS)	Col	28%	±2%	59%	±4%	12%
		S602	27%	±1%	61%	±3%	12%
	T2 (49 DAS)	Col	30%	±2%	38%	±6%	32%
		S602	31%	±2%	33%	±3%	35%
P% ¹⁵ N	T0 (35 DAS)	Col	20%	±7%	80%	±7%	
		S602	19%	±7%	81%	±7%	
	T1 (42 DAS)	Col	22%	±2%	72%	±4%	6%
		S602	26%	±6%	66%	±8%	8%
	T2 (49 DAS)	Col	30%	±9%	53%	±9%	17%
		S602	27%	±7%	50%	±8%	23%

(2004) and showed that *GLN1;2* mRNA was increased by ammonium in roots and also in shoots ([Fig. 10A](#)). The staining of the *pGLN1;2::uidA* transformants after 7 d of growth on nitrate or ammonium medium confirmed that the

Table 2. Nitrogen concentration [N% as mg (100 mg⁻¹ DW)], carbon concentration [C% as mg (100 mg⁻¹ DW)] in dry remains (DR) and seeds, C/N ratio in seeds, partitioning of dry weight (P%DW) and total ¹⁵N (P%¹⁵N) in the seeds are compared between mutant and wild type at high (10 mM) and low (2 mM) nitrate supplies

The partitioning P% (in % of the whole plant) of the DW in seeds is the ratio [(total seed DW in mg) (whole plant DW in mg)] and is also known as harvest index. The partitioning P% (in % of the whole plant) of ¹⁵N in seeds is the ratio [(μg ¹⁵N in the seeds)/(μg ¹⁵N in the whole plant)]. Data have been measured or calculated from eight individual plants cultivated in two growth repeats. Means and SD (*n*=7–8) are presented. No significant differences between WT and mutant can be detected.

Genotypes	Nutrition	N% in DR	C% in DR	N% in seeds	C% in seeds	C/N seeds	P%DW	P% ¹⁵ N
WS	10 mM	4.3±0.2	36.2±1.4	4.3±0.1	56.1±2.0	12.9±0.7	29%±2%	38%±6%
E03	10 mM	4.1±0.2	36.3±1.4	4.5±1.0	57.4±8.0	12.8±1.0	29%±5%	38%±7%
WS	2 mM	0.72±0.03	41.3±1.3	3.2±0.1	57.8±0.8	18.1±0.4	27%±2%	64%±11%
E03	2 mM	0.79±0.12	41.3±0.8	3.2±0.1	57.3±0.3	17.6±0.6	27%±6%	64%±6%

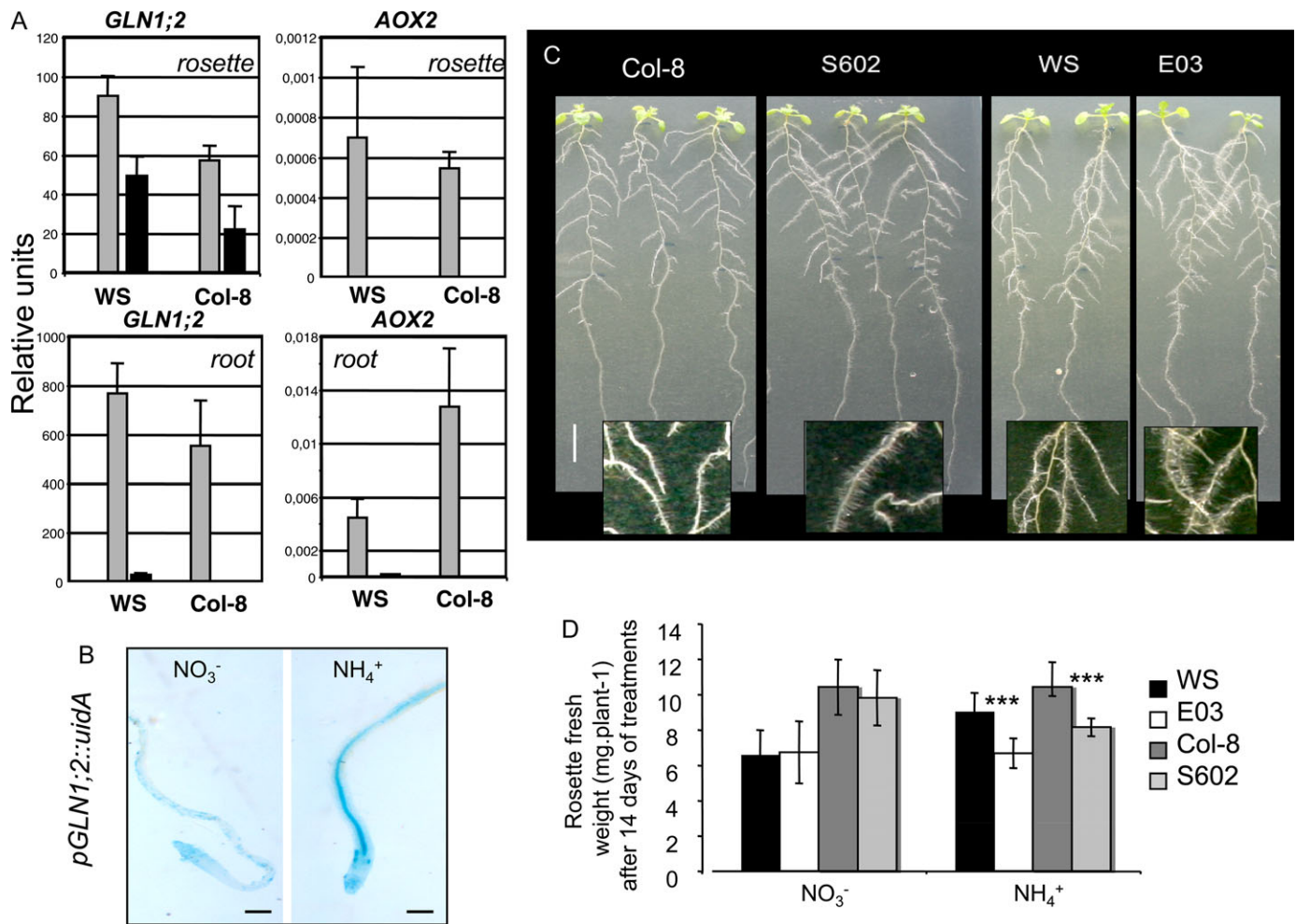


Fig. 10. *In vitro*, growth of *gln1;2* mutant is affected on ammonium-containing medium. Plants were grown *in vitro* on vertical plates with ammonium or nitrate as the sole nitrogen sources (see Materials and methods). (A) *GLN1;2* and *AOX2* (alternative oxidase 2) mRNA steady-state levels are higher in roots of plants grown on ammonium (grey bars) than on nitrate (black bars). Quantitative real-time PCR was performed using *UBQ2* as the control. Means of independent triplicate plate samples and SD values (*n*=3) are indicated; (B) GUS staining of *pGLN1;2::uidA* seedlings was deeper in roots of plants grown with ammonium than in roots of plants grown on nitrate. Black bars indicate 0.1 mm scale. The experiment was repeated twice giving similar results. (C) Under ammonium, the root hair of the mutants is longer than the wild type. Lengths of primary and secondary roots were not significantly different. The experiment was repeated four times giving similar results. The white bar indicates 1 cm scale. (D) Fresh weight of mutant rosettes is significantly lower than wild type when plants are grown on ammonium plates. Such a difference was not observed under nitrate conditions. Means ±SD (*n*=6) are presented and *** indicates significant differences between wild type and mutant (Student *t* test, *P* < 0.001).

activity of the *GLN1;2* promoter is highly enhanced by ammonium in roots (Fig. 10C). The phenotype of the mutants was then examined under similar conditions and revealed that when grown with 5 mM ammonium as the sole nitrogen source, *gln1;2* mutants exhibited longer root hair phenotypes and smaller rosette biomass compared with the wild type (Fig. 10C, D). Such a phenotype suggested that ammonium feeding was more stressful for mutants than wild types and that *GLN1.2* was essential for plants to maintain ammonium homeostasis and plant growth. As a control of ammonium toxic effect on ammonium feed plants, the expression of alternative oxidase *AOX2* was monitored (Escobar *et al.*, 2006). A large increase in *AOX2* mRNA level was observed in ammonium treated plants.

Discussion

Glutamine synthetase gene families have been described in many plant species. Our present work and the data from *gln1* knockout lines of rice, maize, and tobacco (Brugière *et al.*, 1999; Tabuchi *et al.*, 2005; Martin *et al.*, 2006) reveal a specialization of GS1 isoenzyme function depending on leaf tissue and also on plant species. Phylogenetic studies of GS nucleotide or amino acid sequences have shown that chloroplastic and cytosolic GS form two sister groups with a common ancestor and that two genes diverged by duplication before the divergence of gymnosperms/angiosperms (Saez *et al.*, 2000). A new phylogenetic tree established using bayesian inference and based on 93 nucleotide sequences of *GLN* genes corresponding to 42 plant species is presented in Supplementary Fig. S8 at JXB online. The tree was rooted using the *GLN2* group. This establishes a first division between *GLN1* and *GLN2* sequences that confirms previous findings and reflects the presence of 200 nucleotides for a chloroplast signal peptide in the *GLN2* coding sequence. In the *GLN1* clade, all of the branches, for gymnosperms, monocots, and dicots start from the same point. While all monocot *GLN1* are grouped in the same branch, the *GLN1* genes from dicots are divided into different branches. Interestingly, the Brassicaceae has two different *GLN1* groups and the five *GLN1* genes from *Arabidopsis* are divided as follows: *GLN1;1*, *GLN1;2*, and *GLN1;4* are in one cluster and *GLN1;5* and *GLN1;3* in another cluster. A functional redundancy and specialization of the *GLN1* present in the same cluster can be suggested. As such it is interesting to note that *GLN1;1*, *GLN1;2* and *GLN1;4* are the three *Arabidopsis* GS1 genes known to be over-expressed in senescing leaves, and that *GLN1;5* and *GLN1;3* are the two *GLN1* genes known to be mainly expressed in seeds. Due to the strong divergence between all the monocot and the dicot *GLN1* genes, which are divided into different branches, it appeared impossible to use the large set of data obtained from rice or maize mutants to presume the role of the different *GLN1* genes in *Arabidopsis*. By contrast, the strong homologies existing between the *Arabidopsis* and the *Brassica napus* *GLN1*

sequences suggest an easier transfer of knowledge from *Arabidopsis* to *B. napus*.

Among the three *Arabidopsis* *GLN1* genes over-expressed in senescing leaves, *GLN1;2* was the most highly expressed in leaves. Its senescence-related expression has been described for a long time (Bernhard and Matile, 1994). Diaz *et al.* (2008) confirmed that *GLN1;2* is over-expressed with leaf ageing, however Guo *et al.* (2004) showed that the senescence-related increase in the *GLN1;2* expression level is lower for *GLN1;2* than for *GLN1;4* and *GLN1;1*.

While the expression level of *GLN1;2* measured in leaves and roots of wild-type plants was similar to *GLN1;1* and *GLN1;3* expression levels, and much higher than *GLN1;4* (see Supplementary Table S2 at JXB online) it was found that the *gln1;2* mutants contained very weak amounts of GS1 protein in the roots and a large decrease in GS1 protein content in the leaves. In addition, while there is some evidence that the GS2 protein content in leaves of C₃ plants, as detected by Western blot experiments, is much higher than the GS1 protein content (Masclaux *et al.*, 2000; Diaz *et al.*, 2008; Lemaître *et al.*, 2008; this work), the total GS activity was surprisingly decreased by more than 50% in the leaves of our *gln1;2* mutants at high nitrate supply. The lack of rationality between the GS2 and *GLN1;2* protein contents and the relative GS activities in wild type and mutants suggests that either GS1 specific activity is dramatically higher than GS2 specific activity, or that the GS2 protein content observed on Western blots is not fully active. The possibility that part of the GS2 protein is inactivated would be in good agreement with similar discrepancies described in several studies between the decrease of *GLN2* expression, GS2 activity, and GS2 protein content in the same leaf tissue (Masclaux *et al.*, 2000; Diaz *et al.*, 2008). Recently, it was indeed shown that GS2 is regulated by redox through thioredoxin interaction (Lindahl and Kieselbach, 2009).

GLN1;2 is present in companion cells and might play a role in proline concentration in phloem saps and old leaves

The analysis of *GLN1;2* promoter activity showed that *GLN1;2* was mainly expressed in the veins of shoot and stems. Previously, Ishiyama *et al.* (2004) had shown that the *GLN1;2* promoter was active in the root vasculature of *Arabidopsis* using *promoter::GFP* fusions. In the leaves, it was found that the promoter was mainly active in tertiary and quaternary veins of mature leaves. These veins are known to be those where nutrients are uploaded to the phloem for export (Haritatos *et al.*, 2000a, b). The colocalization of antibody-related fluorescence and DAPI allowed us to localize *GLN1;2* more precisely to phloem companion cells. Therefore, a special role for *GLN1;2* in nutrient translocation is strongly suggested. For this reason both amino acid composition in the phloem exudates of leaves and nitrogen remobilization using ¹⁵N tracing were monitored. Phloem exudates of mutant mature leaves were lacking proline compared to the wild type. The only

difference in amino acid content in leaves was an increase of proline, glutamine, and alanine proportions in the six first leaves of mutants grown with 10 mM nitrate. These differences between wild-type and mutant lines, only observed in the 6FL of plants grown at high nitrate supply, increased with ageing. This was in good agreement with the previous finding that *GLN1;2* is over-expressed in old leaves, but it is surprising that no effect can be observed in the other leaves where *GLN1;2* is also highly expressed under high nitrate supply. It can then be hypothesized that, by decreasing their leaf biomass, *gln1;2* mutants have been able to maintain global C and N homeostasis. The fact that total amino acid concentration was increased in the 6FL under high nitrate suggests that the use of amino acids for protein synthesis might have been a limiting point for leaf growth. In addition, the increase of proline in the 6FL might be symptomatic of stress.

Brugière *et al.* (1999) reported that the impairment of phloem GS1 activity resulted in the decrease of proline content in roots, stems, and leaves when ammonium was fed to plants. In our case, it is not clear why proline is increased in 6FL of *gln1;2* and if it is related to ammonium accumulation or to stress. The analysis of the steady-state level of the *GLN1;1*, *GLN1;3*, *GLN1;4*, and *ASN1-ASN3* mRNA levels did not reveal any secondary effects of the *gln1;2* mutation on the expression of other glutamine-related genes (see Supplementary Table S2 at *JXB* online) that might explain the increase in glutamine content. Such a compensatory effect on glutamine content via the GS/AS pathway seems improbable.

gln1;2 mutants are affected in vegetative biomass but not in plant fitness, seeds' C/N ratio, and global N remobilization under ample nitrate supply

While *GLN1;2* is important for rosette biomass and stem growth rate under 10 mM nitrate supply, no defect was observed of the *gln1;2* mutants in yield, harvest index, seeds' C/N or N-remobilization to the seeds. It appears that, at the end of their life cycle, *gln1;2* mutants have completely recovered/adapted and therefore are not different from the wild type. This suggests that the role of *GLN1;2* is especially important at the vegetative stage but is not essential for seed production. Recently, it was shown that, in *Arabidopsis* grown under non-limiting N supply (10 mM nitrate), nitrate influx is lower in the reproductive than in the vegetative stage (Masclaux-Daubresse *et al.*, 2010). Together with the biomass phenotype observed, this suggests that *GLN1;2* operates at the same time as the nitrate primary assimilation process occurs rather than during the N remobilization process associated with seed filling.

In contrast with other GS1 mutants described in maize and rice, our *gln1;2* mutants were only affected in vegetative biomass. In maize, mutation in *gln1-3* or *gln1-4* affects kernel number or size but not vegetative biomass (Martin *et al.*, 2006). In rice, mutants deficient in cytosolic OsGS1;1 had a lower vegetative biomass and a smaller grain yield (Tabuchi *et al.*, 2005).

GLN1;2 might be involved in ammonium detoxification in roots and leaves of *Arabidopsis* under high nitrate supply

Beside the unexpected effect of the *gln1;2* mutation on the total GS activity measured *in vitro*, a good agreement was found between the decrease of total GS activity in the two *gln1;2* mutants under high nitrate supply, the higher ammonium concentrations in the *gln1;2* mutants grown under the same conditions and their lower biomass phenotype (Fig. 11). Altogether, our results show that *GLN1;2* is a key enzyme for shoot biomass when plants are cultivated under ample nitrogen conditions. The fact that the major phenotypes of *gln1;2* were observed at 10 mM nitrate and not at 2 mM is also in good agreement with the low affinity of *GLN1;2* for ammonium reported previously (Ishiyama *et al.*, 2004). The lower biomass of *gln1;2* mutants under high nitrate supply might be due to the fact that the rate of glutamine synthesis is affected and, as a result, the biosynthesis and distribution of all the amino acids in plant are impaired. Another reason for biomass defect would be that

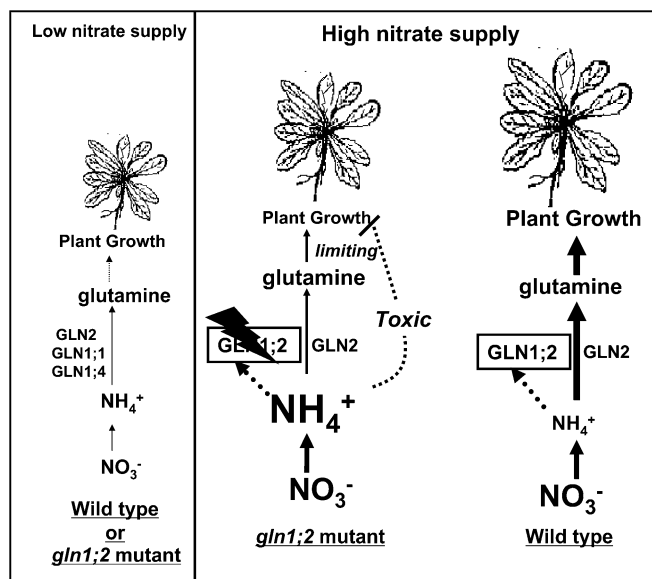


Fig. 11. Schematic model of the potential effect of *gln1;2* mutation on plant growth, nitrogen assimilation, and ammonium detoxification. Under low nitrate supply (on the left), *GLN1;2* expression is low; the glutamine synthetases *GLN2*, *GLN1;4*, and *GLN1;1* are not affected by *gln1;2* mutation and are sufficient to support nitrogen assimilation for plant growth. Therefore phenotype of wild types and mutants are similar. Under high nitrate supply (on the right), *GLN1;2* is essential for plant homeostasis and growth. An explanatory model of the differences in *gln1;2* and wild-type phenotypes is presented. *GLN1;2* that is the most highly expressed *GLN1* under high nitrate supply and that is induced by ammonium (dot line), is missing in *gln1;2* mutants. As a result, ammonium accumulation is increased and the rate of glutamine synthesis is limited. Both the ammonium toxic effect and the glutamine biosynthetic rate are limiting plant growth. Dotted lines represent regulatory controls, dashed lines represent a toxic effect, and plain lines represent metabolic fluxes.

ammonium accumulation is toxic and might increase O₂ respiration rate and thus carbohydrate over-consuming (Escobar *et al.*, 2006). The increase in the alternative oxidase *AOX2* expression observed in our plants under ammonium feeding conditions is consistent with such an hypothesis. In addition, Hachiya *et al.* (2010) recently reported that growing plants with ammonium as a sole source of nitrogen induced respiration in *Arabidopsis* and that the ammonium-dependent induction of O₂ uptake rates is related to ATP consumption via plasma membrane H⁺-ATPase. Such energy consumption to maintain proton balance might also impair plant growth.

The roles of GS1 in plant physiology might be dependent on the magnitude of nitrate supplies.

A striking result is that mutating *GLN1;2* results in a decrease of about half of the total GS activity in leaves when plants are grown at high nitrate supply but not when plants are grown at low nitrate. This feature, which is in good agreement with the vegetative biomass phenotype displayed by the mutants and with the low affinity of *GLN1;2* for ammonium described by Ishiyama *et al.* (2004), suggests that *GLN1;2* is essential for plants under ample N nutrition but not under limiting nitrate supply. The Western blots performed on leaves of wild-type and *gln1;2* mutants grown at low nitrate (Fig. 7) also clearly show that the majority of the GS1 proteins in leaves under low nitrate supply are not encoded by *GLN1;2* and the higher steady-state levels of *GLN1;1* and *GLN1;4* mRNAs monitored at 2 mM nitrate compared with 10 mM nitrate suggest that *GLN1;4* and *GLN1;1* are more important for plant under low nitrate supply (see Supplementary Table S2 at JXB online). The high affinity for ammonium described for *GLN1;1* and *GLN1;4* by Ishiyama *et al.* (2004) is also consistent with a role of *GLN1;4* and *GLN1;1* under N-limiting condition.

Therefore, studying the role of GS1 isoforms necessitates a careful consideration of the plant nutritive conditions in our assays and the role of nitrogen availability in the control of the expression and the activity of each isoform. Characterization of the other members of the *GLN1 Arabidopsis* family is of course needed to understand the complexity of GS1 function in ammonium assimilation and reassimilation at the whole plant level and under fluctuating environments. Recently the fact was illustrated that there is some natural variation in the expression of nitrogen genes in *Arabidopsis* accessions (Masclaux-Daubresse *et al.*, 2010). From the assumption arising from the present study that *GLN1* multigenic families might have a role in plant adaptation to low and high nitrate environments, it would be interesting to map expression QTL for *GLN1* genes and the QTL of biomass features under different nitrate supplies.

Supplementary data

Supplementary data can be found at JXB online.

Supplementary Fig. S1. Changes in expression level (\pm SD) of *GLN1;1*, *GLN1;2*, *GLN1;3*, *GLN1;4* and *GLN1;5* in different organs of *Arabidopsis*.

Supplementary Fig. S2. Changes in expression level (\pm SD) of *GLN1;1*, *GLN1;2*, *GLN1;3*, *GLN1;4* and *GLN1;5* in roots of *Arabidopsis* treated or not with nitrate.

Supplementary Fig. S3. Comparison of GS activities and isoenzymes in rosettes (A) and roots (B) of *gln1;2* E03 mutant (Mut) and WS (WT) after ion-exchange chromatography.

Supplementary Fig. S4. Immuno-localization of GS using indirect immunofluorescence analysis on S602 (A, B, C), Col-8 (D, E, F), E03 (G, H, I) and WS (J, K, L).

Supplementary Fig. S5. Individual amino acid compositions in the new leaves (NL) (given as the percentage of total amino acid) of WS wild-type (black bars) and E03 mutant (white bars) grown at 2 mM (A, B, C, D) and 10 mM (E, F, G, H) nitrate.

Supplementary Fig. S6. Individual amino acid compositions in the six first leaves (6FL) (given as the percentage of total amino acid) of WS wild-type (black bars) and E03 mutant (white bars) grown at 2 mM (A, B, C, D) and 10 mM (E, F, G, H).

Supplementary Fig. S7. Individual amino acid compositions in the phloem exudates (given as the percentage of total amino acid) were determined at 42 DAS (A) and 66 DAS (B) for WS wild-type (black bars) and E03 mutant (white bars) plants.

Supplementary Fig. S8. Rooted phylogenetic tree of the GLN gene family from several plant species.

Supplementary Table S1. PCR primers used for qPCR.

Supplementary Table S2. Steady-state amounts of *GLN2*, *GLN1;1*, *GLN1;2*, *GLN1;3*, *GLN1;4*, *GLN1;5*, *ASN1*, and *ASN2*, *ASN3* mRNA in the rosettes (A) and roots (B) of S602 *gln1;2* mutant and wild type quantified by quantitative RT-PCR using primers described in Table S1.

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References

- Bernhard WR, Matile P. 1994. Differential expression of glutamine synthetase genes during senescence of *Arabidopsis thaliana* rosette leaves. *Plant Science* **98**, 7–14.
- Brugière N, Limami A, Lelandais M, Roux Y, Sangwan RS, Hirel B. 1999. Glutamine synthetase in the phloem plays a major role in controlling proline production. *The Plant Cell* **11**, 1995–2012.
- Cabello P, Agüera E, de la Haba P. 2006. Metabolic changes during natural ageing in sunflower (*Helianthus annuus*) leaves:

expression and activity of glutamine synthetase isoforms are regulated differently during senescence. *Physiologia Plantarum* **128**, 175–185.

Clough S, Bent A. 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *The Plant Journal* **16**, 735–743.

Diaz C, Lemaître T, Christ C, Azzopardi M, Kato Y, Sato F, Morot-Gaudry J, Le Dily F, Masclaux-Daubresse C. 2008. Nitrogen recycling and remobilization are differentially controlled by leaf senescence and development stage in *Arabidopsis* under low nitrogen nutrition. *Plant Physiology* **147**, 1437–1449.

Diaz C, Purdy S, Christ A, Morot-Gaudry JF, Wingler A, Masclaux-Daubresse C. 2005. Characterization of markers to determine the extent and variability of leaf senescence in *Arabidopsis*. A metabolic profiling approach. *Plant Physiology* **138**, 898–908.

Escobar MA, Geisler DA, Rasmusson AG. 2006. Reorganization of the alternative pathways of the *Arabidopsis* respiratory chain by nitrogen supply: opposing effects of ammonium and nitrate. *The Plant Journal* **45**, 775–788.

Fuentes S, Allen D, Ortiz-Lopez A, Hernandez G. 2001. Over-expression of cytosolic glutamine synthetase increases photosynthesis and growth at low nitrogen concentrations. *Journal of Experimental Botany* **52**, 1071–1081.

Gifford ML, Dean A, Gutierrez RA, Coruzzi GM, Birnbaum KD. 2008. Cell-specific nitrogen responses mediate developmental plasticity. *Proceedings of the National Academy of Sciences, USA* **105**, 803–808.

Guo Y, Cai Z, Gan S. 2004. Transcriptome of *Arabidopsis* leaf senescence. *Plant, Cell and Environment* **27**, 521–549.

Habash D, Massiah A, Rong H, Wallsgrove R, Leigh R. 2001. The role of cytosolic glutamine synthetase in wheat. *Annals of Applied Biology* **138**, 83–89.

Hachiya T, Watanabe CK, Takahara K, Kawaiyamada M, Uchimiya H, Uesono Y, Terashima I, Noguchi K. 2010. Ammonium-dependent respiratory increase is dependent on cytochrome pathway in *Arabidopsis thaliana* shoots. *Plant, Cell and Environment* in press.

Haritatos HE, Ayre B, Turgeon R. 2000a. Identification of phloem involved in assimilate loading in leaves by the activity of the galactinol synthase promoter. *Plant Physiology* **123**, 929–937.

Haritatos E, Medville R, Turgeon R. 2000b. Minor vein structure and sugar transport in *Arabidopsis thaliana*. *Planta* **211**, 105–111.

Hirel B, Bertin P, Quillere I, et al. 2001. Towards a better understanding of the genetic and physiological basis for nitrogen use efficiency in maize. *Plant Physiology* **125**, 1258–1270.

Ishiyama K, Inoue E, Watanabe-Takahashi A, Obara M, Yamaya T, Takahashi H. 2004. Kinetic properties and ammonium-dependent regulation of cytosolic isoenzymes of glutamine synthetase in *Arabidopsis*. *Journal of Biological Chemistry* **279**, 16598–16605.

Jing Z, Gallardo F, Pascual M, Sampalo R, Romero J, Torres de Navarra A, Canovas F. 2004. Improved growth in a field trial of transgenic hybrid poplar over-expressing glutamine synthetase. *New Phytologist* **164**, 137–145.

Leegood R, Lea P, Adcock M, Hausler R. 1995. The regulation and control of photorespiration. *Journal of Experimental Botany* **46**, 1397–1414.

Lemaître T, Gaufichon L, Boutet-Mercey S, Christ A, Masclaux-Daubresse C. 2008. Enzymatic and metabolic diagnostic of nitrogen deficiency in *Arabidopsis thaliana* Wassileskija accession. *Plant and Cell Physiology* **49**, 1056–1065.

Li MG, Villemur R, Hussey PJ, Silflow CD, Gantt JS, Snustad DP. 1993. Differential expression of six glutamine synthetase genes in *Zea mays*. *Plant Molecular Biology* **23**, 401–440.

Lindahl M, Kieselbach T. 2009. Disulphide proteomes and interactions with thioredoxin on the track towards understanding redox regulation in chloroplasts and cyanobacteria. *Journal of Proteomics* **72**, 416–438.

Martin A, Belastegui-Macadam X, Quillere I, Floriot M, Valadier MH, Pommel B, Andrieu B, Donnison I, Hirel B. 2005. Nitrogen management and senescence in two maize hybrids differing in the persistence of leaf greenness: agronomic, physiological and molecular aspects. *The New Phytologist* **167**, 483–492.

Martin A, Lee J, Kichey T, et al. 2006. Two cytosolic glutamine synthetase isoforms of maize are specifically involved in the control of grain production. *The Plant Cell* **18**, 3252–3274.

Masclaux C, Valadier M, Brugière N, Morot-Gaudry J, Hirel B. 2000. Characterization of the sink/source transition in tobacco (*Nicotiana tabacum* L.) shoots in relation to nitrogen management and leaf senescence. *Planta* **211**, 510–518.

Masclaux-Daubresse C, Daniel-Vedele F, Dechorgnat J, Chardon F, Gaufichon L, Suzuki A. 2010. Nitrogen uptake, assimilation and remobilisation in plants: challenges for sustainable and productive agriculture. *Annals of Botany* (Special Issue of Plant Nutrition) **105**, 1141–1157.

Masclaux-Daubresse C, Reisdorf-Cren M, Pageau K, Lelandais M, Grandjean O, Kronenberger J, Valadier MH, Feraud M, Jougllet T, Suzuki A. 2006. Glutamine synthetase-glutamate synthase pathway and glutamate dehydrogenase play distinct roles in the sink-source nitrogen cycle in tobacco. *Plant Physiology* **140**, 444–456.

Obara M, Sato T, Sasaki S, Kashiba K, Nagano A, Nakamura I, Ebitani T, Yano M, Yamaya T. 2004. Identification and characterization of a QTL on chromosome 2 for cytosolic glutamine synthetase content and panicle number in rice. *Theoretical and Applied Genetics* **110**, 1–11.

Oliveira I, Brears T, Knight T, Clark A, Coruzzi G. 2002. Over-expression of cytosolic glutamine synthetase. Relation to nitrogen, light, and photorespiration. *Plant Physiology* **129**, 1170–1180.

Purnell M, Botella J. 2007. Tobacco isozyme 1 of NAD(H)-dependent glutamate dehydrogenase catabolizes glutamate *in vivo*. *Plant Physiology* **143**, 530–539.

Sakakibara H, Kawabata S, Hase T, Sugiyama T. 1992. Differential effect of nitrate and light on the expression of glutamine synthetase and ferredoxin-dependent glutamate synthase in maize. *Plant and Cell Physiology* **33**, 1193–1198.

- Sakakibara H, Shimizu H, Hase T, Yamazaki Y, Takao T, Shimonishi Y, Sugiyama T.** 1996. Molecular identification and characterization of cytosolic isoforms of glutamine synthetase in maize roots. *Journal of Biological Chemistry* **271**, 29561–29568.
- Sàez CÀ, Muñoz-Chapuli R, Plomion C, Frigerio J-M, Cànovas FM.** 2000. Two genes encoding distinct cytosolic glutamine synthetases are closely linked in the pine genome. *FEBS letters* **477**, 237–243.
- Tabuchi M, Sugiyama K, Ishiyama K, Inoue E, Sato T, Takahashi H, Yamaya T.** 2005. Severe reduction in growth rate and grain filling of rice mutants lacking OsGS1;1, a cytosolic glutamine synthetase1;1. *The Plant Journal* **42**, 641–651.
- Wallsgrave R, Turner J, Hall N, Kendall A, Bright S.** 1987. Barley mutants lacking chloroplast glutamine synthetase-biochemical and genetic analysis. *Plant Physiology* **83**, 155–158.