Glutamine synthetase (GS; EC 6.3.1.2) is a key enzyme of nitrogen assimilation, catalyzing the synthesis of glutamine from ammonium and glutamate. In Arabidopsis, cytosolic GS (GS1) was accumulated in roots when plants were excessively supplied with ammonium; however, the GS activity was controlled at a constant level. The discrepancy between the protein content and enzyme activity of GS1 was attributable to the kinetic properties and expression of four distinct isoenzymes encoded by GLN1;1, GLN1;2, GLN1;3 and GLN1;4, genes that function complementary to each other in Arabidopsis roots. GLN1;2 was the only isoenzyme significantly up-regulated by ammonium, which correlated with the rapid increase in total GS1 protein. GLN1;2 was localized in the vasculature and exhibited low affinities to ammonium (K_m = 2450 ± 150 μM) and glutamate (K_m = 3.8 ± 0.2 mM). The expression of the counterpart vascular tissue-localizing low affinity isoenzyme, GLN1;3, was not stimulated by ammonium; however, the enzyme activity of GLN1;3 was significantly inhibited by a high concentration of glutamate. By contrast, the high affinity isoenzyme, GLN1;1 (K_m for ammonium < 10 μM, K_m for glutamate = 1.1 ± 0.4 mM) was abundantly accumulated in the surface layers of roots during nitrogen limitation and was down-regulated by ammonium excess. GLN1;4 was another high affinity-type GS1 expressed in nitrogen-starved plants but was 10-fold less abundant than GLN1;1. These results suggested that dynamic regulations of high and low affinity GS1 isoenzymes at the levels of mRNA and enzyme activities are dependent on nitrogen availabilities and may contribute to the homeostatic control of glutamine synthesis in Arabidopsis roots.

Glutamine synthetase (GS; EC 6.3.1.2) is responsible for the primary assimilation of ammonium in higher plants (1–4). Ammonium is assimilated into glutamine and glutamate through a consecutive reaction of GS and glutamate synthase (GOGAT), the so-called GS/GOGAT cycle. Plants have two types of GS isoenzymes that localize in different compartments: one located in the cytosol (GS1) and the other in the plastid/chloroplasts (GS2) (1–4). GS1 is the major form of GS in plant roots, and the ammonium taken up from the soil is directly converted to Gln by its reaction. Molecular biological studies have identified a number of genes encoding GS1 from various plant species (5–9). The presence of multiple GS1 isoenzymes complicates the overall understanding of their physiological functions. The isoenzymes of GS1 show organ- and cell-specific patterns of expression and are developmentally regulated (10–17). In addition, the expression of GS1 is metabolically regulated by the availability of nitrogen and carbon sources (18–22, 24).

In the roots of legumes, GS1 is regulated by ammonium supplied from the environment or the symbiotic nitrogen fixation (18–21). Transgenic studies with the soybean GS1 promoter suggested that ammonium-dependent regulation is specific for nitrogen assimilation in leguminous plants; the soybean-derived GS1 promoter was not able to display the ammonium-induced expression of reporter activity in tobacco (20). This poses questions of whether the stimulation of GS1 expression by ammonium is genuinely associated with symbiosis and whether diotyledonous plants are furnished or not furnished with the regulatory mechanisms that may perceive the ammonium signal. The availability of ammonium also caused changes in the expression of some of the GS1 genes in non-leguminous plants. For example, among the five isoenzymes of GS1 (GS1a, GS1b, GS1c, GS1d, and GS1e) identified from maize (9, 13), the mRNA of GS1c and GS1d accumulated following application of ammonium to roots, whereas those of GS1a and GS1b were suppressed under the same condition (22).

In Arabidopsis, five putative genes for GS1, GLN1;1, GLN1;2, GLN1;3, GLN1;4, and GLN1;5, are encoded in the genome (23). Oliveira and Coruzzi (24) reported that the application of sucrose to the medium causes induction of GLN1;1, GLN1;2, and GLN1;3 mRNAs and that amino acids attenuated the effect of sucrose, suggesting that a metabolic regulation of GS1 is associated with the relative abundance of carbon skeleton versus amino acids accumulated in the root tissue. These results suggest a negative feedback regulation of GS1 by Gln or the downstream nitrogen metabolites as in the case of ammonium transporters. However, the exact functional roles and physiological diversities of the individual GS1 isoenzymes in Arabidopsis have not been well characterized.

In the present study, we determined the kinetic properties and ammonium-dependent response of the Arabidopsis GS1 isoenzymes. We demonstrated that GS1 expressed in roots (GLN1;1, GLN1;2, GLN1;3, and GLN1;4) can be classified into two distinct groups, the high affinity and low affinity enzymes localized at the root surface and vasculature, respectively. The
characteristics of the GS1 isoforms in Arabidopsis were fitted into the negative regulatory pathways of ammonium influx and assimilation, which is apparently different from those operatively positive for the active assimilation of externally supplied ammonium in the roots of legumes and maize. The results presented here clearly demonstrated that the multiplicity of cytosolic GS1 in higher plants does not simply provide functional redundancy but, rather, confers specified roles to the individual isoforms bearing distinctive kinetic properties and ammonium responsiveness. This is the first report presenting the complete and precise data of enzymatic properties and cell type-specific localization of GS1 isoforms in higher plants.

EXPERIMENTAL PROCEDURES

Plant Culture Conditions—Arabidopsis thaliana ecotype Columbia (Col-0) was used for all experiments. Plants were cultured in a growth chamber controlled at 22 °C with 60% relative humidity under 16-h light and 8-h dark cycles. The light intensity was 40 μmol m^{-2} s^{-1}. Plants were grown under sterile conditions on agar medium containing 7 g/L. Arabinose, 1 mg/L. Salkiewicz (28) was grown in Murashige and Skoog liquid medium supplemented with 2.5 mM potassium phosphate and 4.5 μM 2,4-dichlorophenoxyacetic acid by continuous shaking under dark conditions.

Cloning of Arabidopsis GLN1 cDNAs—Molecular biological experiments were carried out according to the standard protocols (27). The coding sequences of GLN1 cDNAs encoding GS1 were isolated by reverse transcriptase PCR. Primers were designed according to the annotations of the nucleotide sequence of Arabidopsis GLN1 gene (23) presented in The Institute for Genomic Research (www.tigr.org/tdb/e2k1/ath1) and the Munich Information Center for Protein Sequences (mips.gsf.de/proj/ath1/index.html) databases. The locus numbers for GLN1 genes are At1g36780 (GLN1;1), At1g68200 (GLN1;2), At3g17800 (GLN1;3), At5g18570 (GLN1;4), and At5g8470 (GLN1;5). Total cDNA was extracted using the RNeasy plant mini kit (Qiagen, Hilden, Germany). Reverse transcription and PCR were carried out using Omniscript reverse transcriptase (Qiagen) and KOD plus DNA polymerase (Toyobo, Tokyo, Japan). The amplified PCR products were cloned into pCR-Blunt II-TOPO (Invitrogen, Carlsbad, CA) and fully sequenced.

Quantitative Real Time PCR Analysis—Extraction of RNA and cDNA synthesis was carried out as described above for cDNA cloning. Gene-specific primers are presented in Table I. Constitutive expression of GS1 was determined by using the quantitative real-time PCR analysis. GS1 expression was confirmed in parallel (Table I). The PCR products were detected and quantified as SYBR Green fluorescence (Applied Biosystems, Foster City, CA) using the Gene Amp 5700 sequence detection system (Applied Biosystems). The mRNA content was quantitatively determined by using a purified cDNA clone as a standard for its calibration.

Preparation of Anti-GS1 Antibody—The cDNA fragment encoding rice GS1, RGS28 (GenBank™ accession number X14245) (7) was inserted into the NcoI site of pTrc99A (Amersham Biosciences). The nucleotide sequence downstream of the translation initiation codon was modified to an AT-rich structure to obtain high expression of the recombinant GS1 protein (22). The first 240 bp portion of the RGS28 coding sequence was deleted by HinclI digestion and replaced with the oligonucleotide 5'-TCAGCAAGCTTGGTACTTACAGTCGTCG-3' without changing the encoding amino acid residues. The 3'-end of RGS28 was digested with PstI and ligated with the oligonucleotide 5'-CTGGAAGCGCCATCATCATTCATATGACCTG-3', adding His codons and a stop codon at the C terminus. The modified RGS28 was cloned in pTrc99A and transformed to E. coli K12 JM109. Culture of E. coli and purification of the recombinant protein were performed as described previously (22). To prepare polyclonal antibody against the rice recombinant GS1 protein, 500 μg of the purified antigen emulsified with adjuvant was injected into a rabbit. Additional injections (500 μg each) were done twice every 2 weeks. Whole blood was obtained from the carotid artery, and the antisera was collected by centrifugation.

Western Blot Analysis—Proteins were separated in 12.5% (w/v) polyacrylamide gels and transferred to a nitrocellulose membrane (Bio-Rad) by electroblotting. The amount of proteins applied to the gel is indicated in the figure legends. The membrane was incubated with the anti-GS1 polyclonal antibody and goat anti-rabbit IgG alkaline phosphatase conjugate (Promega, Madison, WI) as described previously (28). Proteins cross-reacting with the antibodies were visualized using 5-bromo-4-chloro-3-indolyl phosphate-p-toluidine and nitroblue tetrazo- lithium chloride (Promega).

GS Enzyme Assay—The Gln synthetic activity of GS was determined by quantifying t-Gln synthesized from ammonium and t-Glu (28). A 0.1-ml reaction mixture contained 100 mM tricine-HCl (pH 7.8), 80 mM t-Glu, 6 mM ammonium, 8 mM ATP, 20 mM MgSO4, and 8 mM 2-mercaptoethanol. The amount of proteins used for the enzyme reaction is indicated in Figs. 2 and 6 and Table II. The reaction mixture was incubated at 30 °C for 15 min, terminated by heating at 96 °C for 2 min, and filtered through Ultra-free-MC 5000 NMWL Filter Unit (Millipore, Bedford, MA). Five microliters of filtrate was incubated with AccQ Fluor reagent (Waters, Milford, MA), and the resulting AccQ-derivative of Gln was measured by Waters 2695 high performance liquid chromatography system on an AccQ-Tag column (3.9 mm × 150 mm length; Waters) according to the manufacturer’s instructions.

Extraction of Proteins from Arabidopsis Plants—Approximately 0.2 g of frozen root or leaf tissues were homogenized in an equal volume of GS extraction buffer (50 mM Tris-HCl pH 7.6, 10 mM MgCl2, 10 mM 2-mercaptoethanol, and 5% (w/v) polyvinylpyrrolidone) (28) using a chilled mortar and pestle or with MM-300 mixer mill (Qiagen). The homogenates were centrifuged at 20,000 × g for 10 min under 4 °C. The supernatants were immediately desalted on PD-10 (Amersham Biosciences) or Probe Quant G-50 Micro Columns (Amersham Biosciences) equilibrated with buffer A (25 mM Tris-HCl, pH 7.6, 10 mM MgCl2, and 10 mM 2-mercaptoethanol). The crude enzyme fractions obtained were stored at -80 °C. Protein content was determined by the Coomassie Blue dye binding method (Bio-Rad) using bovine serum albumin as a standard.

Purification of Native GS1 Protein from Arabidopsis Cell Culture—All of the steps of enzyme purification were performed in a cold room (4 °C). One hundred fifty grams of cultured cells were homogenized in 500 ml of GS extraction buffer using a chilled warring blender (28). The homogenate was centrifuged at 27,000 × g for 15 min. The supernatant was fractionated by (NH4)2SO4 precipitation between 30 and 60% saturation. The precipitate was suspended in 50 mM (NH4)2SO4, precipitated by (NH4)2SO4, and centrifuged at 27,000 × g for 15 min. The precipitate was suspended in 2 ml of buffer A and loaded for gel filtration chromatography on a HiLoad 26/60 Superdex 200 pg (32 ml bed volume) (Amersham Biosciences) equilibrated with buffer A. The fractions containing GS activities were further purified by anion exchange chromatography using a HiTrap Q HP (1-ml bed volume) (Amersham Biosciences) equilibrated with buffer A. Protein was eluted by a linear gradient of NaCl from 0 to 1 M.

Expression of Recombinant GS1 Proteins in E. coli—The KpnI and SalI sites were created on the 5' and 3'-ends of the coding region of GLN1 cDNAs by PCR using ROD-F and ROD-R primers (Table II). The amplified PCR products were cloned into pCR-Blunt II-TOPO (Invitrogen) and fully sequenced. The cDNA insert was digested by KpnI and SalI and ligated into the KpnI and SalI sites of pQE2 (Qiagen) to produce the His fusion constructs, termed pQE-GLN1;1, pQE-GLN1;2, pQE-GLN1;3, and pQE-GLN1;4, respectively. E. coli HB101 was transformed with the recombinant construct and grown at 30 °C in 300 ml of LB medium containing 1 x sorbitol and 100 μg/ml ampicillin. When A660 reached 0.5, the culture was cooled down to 20 °C. One millimolar isopropyl-1-thio-β-galactopyranoside was supplied to induce the expression of recombinant GS1 proteins, and the culture was further incubated at 20 °C for 12 h by continuous shaking (180 rpm).

Purification of Recombinant GS1 Enzymes—One volume of E. coli cells collected by centrifugation was resuspended in 0.1 ml of lysis buffer (50 mM NaH2PO4, pH 8, 300 mM NaCl, and 10 mM imidazole). Cell suspensions were incubated for 30 min at 4 °C with lysozyme (Nacalai Tesque, Kyoto, Japan) at the final concentration of 1 mg ml^{-1}. After ultrasonication, the insoluble fraction was removed by centrifugation. The His-tagged protein in the soluble fraction was affinity-purified using a nickel-nitrilotriacetic acid agarose column (1-ml bed volume) (Qiagen). Washing was carried out with a buffer containing 50 mM NaH2PO4, pH 8, 300 mM NaCl, and 20 mM imidazole. Protein was eluted with the same buffer containing 250 mM imidazole. The eluates were desalted and equilibrated in buffer A using PD-10 (Amersham Biosciences). Protein was further purified by anion exchange chromatography with a HiTrap Q HP (1-ml bed volume) (Amersham Biosciences) using the AKTA Prime fast protein liquid chromatography system (Amersham Biosciences). Protein was eluted by 10 ml of a linear NaCl gradient from 0 to 1 M, and the protein peak fractions were analyzed for the presence of a single band of recombinant GS1 protein.
subunit proteins, respectively (Fig. 1B).

Increased Accumulation of GS1 Protein by Ammonium Treatment Is Not Correlated with the GS Activity—The enzyme activity and the abundance of GS1 polypeptides were simultaneously monitored during a time course of ammonium treatment. Two week-old plants were deprived of nitrogen for 3 days and treated with 10 mM ammonium chloride. Root tissues were harvested for Western blotting and GS enzyme assays (Fig. 2). The amount of GS1 protein was significantly higher in the NH4+-treated plants than in the control without nitrogen (Fig. 2, A and B). The abundance of GS1 protein was increased 3-fold within 6 h after transferring the plants to ammonium excess conditions (Fig. 2B). However, under the same condition, the activity of GS was not changed remarkably (Fig. 2C). No significant differences were observed between the GS activities of the ammonium-treated and control plants.

The mRNA contents of individual GS1 isoenzymes were quantified by real-time PCR under nitrogen limitation and ammonium excess (Fig. 3). The gene-specific primer pairs that specifically amplify GLN1 cDNAs are described in Table I. GLN1;1, GLN1;2, GLN1;3, and GLN1;4 were expressed in roots, but the GLN1;5 transcript was not detected. In addition, microarray analysis for Arabidopsis root reported that a signal value for the GLN1;5 mRNA level indicates an “absent” call (35). Thus, we quantified the expression of the four GLN1 genes, GLN1;1, GLN1;2, GLN1;3, and GLN1;4, in roots. Among them, GLN1;1 mRNA was abundantly expressed in roots after 3 days of nitrogen deprivation (Fig. 3A). The amounts of GLN1;1, GLN1;3, and GLN1;4 mRNAs decreased −0.5, 0.7, and 0.3-fold, respectively, after the application of 10 mM ammonium to the medium (Fig. 3B). Conversely, GLN1;2 mRNA was increased −5-fold by ammonium (Fig. 3B) and maintained at high levels during 24 h of ammonium treatment (Fig. 3A). The time course increase in GLN1;2 mRNA (Fig. 4A) corresponded with the accumulation of GS1 protein detected on the Western blot (Fig. 2A). These results suggest that the accumulation of GS1 protein under ammonium excess conditions is associated with the increased expression of GLN1;2 mRNA.

As shown in Figs. 2–4, the increase in GLN1;2 mRNA and the corresponding GS1 protein contents was not correlated with the total GS activities in Arabidopsis roots. To clear these discrepancies, we determined the enzymatic properties and cell type-specific localization of GS1 isoenzymes in Arabidopsis roots.

Purification of Individual Arabidopsis GS1 Isoenzyme—GLN1;1, GLN1;2, GLN1;3, and GLN1;4 were overexpressed in E. coli as His-tagged enzymes and purified to near homogeneity. A native GS1 protein was purified from Arabidopsis cell culture for reference. Coomassie Blue staining of SDS-PAGE

RESULTS

Cytosolic GS1 Is the Major Form in Arabidopsis Roots—The mRNA and protein contents of GS1 and GS2 in Arabidopsis were determined by real-time PCR and Western blot analyses (Fig. 1). Arabidopsis plants were grown on agar medium containing 7 mM nitrate as a sole nitrogen source for 14 days. The total GS1 mRNA was calculated as a sum of GLN1;1, GLN1;2, GLN1;3, GLN1;4, and GLN1;5 mRNAs. The gene-specific primer pairs that specifically amplify the transcripts of individual GS isoenzymes are described in Table I. The GS1 mRNA was −20-fold more abundant than GS2 mRNA in Arabidopsis roots (Fig. 1A). Western blot analysis indicated accumulation of a 40-kDa protein that corresponds to the molecular size of GS1 in roots (Fig. 1B). GS2 (44 kDa) was hardly detectable in roots (Fig. 1B). The results indicate that GS2 mRNA is weakly expressed in roots as a minor GS isoenzyme; however, judging from the Western blots, the contribution of GS2 to the Gln synthesis is not appreciable as compared with GS1. Thus, we concluded that GS1 is the major isoenzyme that carries out the ammonium assimilation in Arabidopsis roots. In leaves, the mRNAs and proteins for both isoenzymes were expressed. On real time PCR, GS2 mRNA was slightly more abundant than GS1 mRNA in leaves (Fig. 1A). Western blot showed two major bands corresponding to the molecular sizes of GS2 and GS1

FIG. 1. GS mRNAs and polypeptides in Arabidopsis roots and leaves. A, GS mRNA contents quantified by real time PCR. Arabidopsis plants were grown for 2 weeks on agar medium containing 7 mM nitrate. Real time PCR was carried out on first strand cDNA synthesized from total RNA as described under “Experimental Procedures.” B, accumulation of GS polypeptides detected by the anti-rice GS1 antibody. Western blot analysis was carried out against 5 μg of total soluble proteins extracted from root (R) and leaf (L) tissues.

(40 kDa) on a Coomassie Blue-stained polyacrylamide gel. The Gln biosynthetic activities of the pooled fractions were assayed to determine the kinetic properties.

Construction of GLN1 Promoter-Green Fluorescent Protein (GFP) Plants—The fusion gene constructs of Arabidopsis GLN1 gene promoters and EGFP (Clontech) for plant transformation were constructed as follows. The 2463-bp, 2501-bp, 1213-bp, and 1161-bp 5′-promoter regions of GLN1;1, GLN1;2, GLN1;3, and GLN1;4, respectively, were amplified from Col-0 Arabidopsis genomic DNA by PCR using KOD plus DNA polymerase (Toyobo). The BamHI site was created on the 5′-end of the forward primer. The reverse primer containing the NcoI restriction site (CCATGG) was created at the translation initiation site of GLN1 gene to make the promoter-GFP fusion. The amplified PCR products were cloned into pCR-Blunt II-TOPO (Invitrogen) and fully sequenced. The resultant GLN1 promoter:EGFP:nopaline synthase terminator cassette was placed in the position of the β-glucuronidase and nopaline synthase terminator in the binary plasmid, pBI101 (Clontech). The binary plasmids were transferred to Agrobacterium tumefaciens GV3101 (pMP90) (30) by the freeze-thaw method (31). Arabidopsis plants were transformed according to the floral dip method (32). Transgenic plants were selected on GM medium (33) containing 50 mg liter−1 kanamycin sulfate. Fluorescence of GFP in transgenic plants was observed under a BX61 microscope equipped with a FV500 confocal laser scanning system and a 505–525-nm band pass filter (Olympus, Tokyo, Japan), as described previously (34).
of GS1 in Arabidopsis roots. As shown in Table II, the four GLN1 isoenzymes of GS1 expressed in Arabidopsis roots can be classified into two major groups by the affinities to substrates. As for Glu, GLN1;1 and GLN1;4 again showed substantially high substrate affinities. The $K_m$ values of GLN1;1 and

**TABLE I**

Gene specific primers used for the real-time PCR analysis

| Name       | Gene     | Sequence                  |
|------------|----------|---------------------------|
| Gln1;1-RF  | GLN1;1   | 5′-CTACACCTTTACTCTGACACTCCACCT-3′ |
| Gln1;1-RR  | GLN1;1   | 5′-CTACACCTTTACTCTGACACTCCACCT-3′ |
| Gln1;2-RF  | GLN1;2   | 5′-CTCTTATGCTCTCCAGTCCAGGACCAG-3′ |
| Gln1;2-RR  | GLN1;2   | 5′-CTCTTATGCTCTCCAGTCCAGGACCAG-3′ |
| Gln1;3-RF  | GLN1;3   | 5′-TTCTCCTTCTTTTACCTTACCCAGGACCAG-3′ |
| Gln1;3-RR  | GLN1;3   | 5′-TTCTCCTTCTTTTACCTTACCCAGGACCAG-3′ |
| Gln1;4-RF  | GLN1;4   | 5′-CTACACCTTTACTCTGACACTCCACCT-3′ |
| Gln1;4-RR  | GLN1;4   | 5′-CTACACCTTTACTCTGACACTCCACCT-3′ |
| Gln1;5-RF  | GLN1;5   | 5′-CTCTTATGCTCTCCAGTCCAGGACCAG-3′ |
| Gln1;5-RR  | GLN1;5   | 5′-CTCTTATGCTCTCCAGTCCAGGACCAG-3′ |
| Gln2-RF    | GLN2     | 5′-TTCTCCTTCTTTTACCTTACCCAGGACCAG-3′ |
| Gln2-RR    | GLN2     | 5′-TTCTCCTTCTTTTACCTTACCCAGGACCAG-3′ |
| 144F       | UBQ2     | 5′-CCAGGTCTGTGAGCGGCTGAAGAAGAAG-3′ |
| 372R       | UBQ2     | 5′-CCAGGTCTGTGAGCGGCTGAAGAAGAAG-3′ |

* The pairs of forward (RF or F) and reverse (RR or R) primers were used to specifically amplify GLN1 and UBQ2 cDNAs.

![Image](99x301 to 266x560)

**FIG. 2.** Effects of ammonium on the activities and accumulation of GS1 in Arabidopsis roots. A. Western blot analysis of GS1 protein contents in Arabidopsis roots during a time course of ammonium treatment. Two week-old Arabidopsis plants were subjected to nitrogen starvation for 3 days prior to the treatment and then transferred to the medium without nitrogen (−N) or with 10 mM ammonium chloride (−NH₄⁺). One microgram of soluble proteins extracted from the root tissues were loaded onto each lane of an SDS-polyacrylamide gel and detected by the anti-rice GS1 antibody. B, the immuno-reacted GS1 protein in panel A was visualized and quantified using Image J (nih.info.nih.gov/ij). Dashed line and solid line correspond to no nitrogen (−N) and 10 mM ammonium chloride treatments, respectively. C, GS activities in Arabidopsis roots. Plants were treated with no nitrogen (dashed line) or 10 mM ammonium chloride (solid line) as indicated in panel A. Ten micrograms of protein was incubated in a 0.1 ml assay mixture as described under “Experimental Procedures.” Means of independent triplicate samples and S.D. values (n = 3) are indicated.

indicates a single polypeptide band in the purified fraction that corresponds to the molecular size of GS1 (Fig. 5A). The purified proteins equally cross-reacted with the anti-rice GS1 antibody, which shows nearly the same affinities against the four Arabidopsis GS1 isoenzymes (Fig. 5B).

Plant GS proteins are made up of eight subunits and have molecular masses ranging from 320 to 380 kDa (1). To determine whether recombinant GS1 enzymes are properly assembled in an octameric structure, the molecular masses of the purified GS1 proteins were estimated under a native condition by gel filtration chromatography calibrated with protein markers of a known size (Fig. 5C). Both of the GS1 proteins purified from the cell culture and recombinant systems showed a similar molecular size of 340–350 kDa, which is consistent with the octameric composition of ~40 kDa subunits (Fig. 5C). From these results, we concluded that the recombinant enzymes are functionally equivalent to the native GS protein derived from Arabidopsis cell culture.

The Arabidopsis GS1 Isoenzymes Exhibit Different Kinetic Properties—The kinetic properties for ammonium, glutamate, and ATP were determined by assaying the Gln biosynthetic activities of individual GLN1 isoenzymes as described under “Experimental Procedures.” As shown in Table II, the four isoenzymes of GS1 expressed in Arabidopsis roots can be classified into two major groups by the affinities to substrates. GLN1;1 exhibited an extremely high affinity to ammonium ($K_m < 10 \mu M$). GLN1;4 also showed a relatively high affinity to ammonium ($K_m = 48 \pm 6.0 \mu M$). By contrast, the affinities of GLN1;2 and GLN1;3 to ammonium were lower than those of GLN1;1 and GLN1;4 by two orders ($K_m$ values; 2450 ± 150 μM for GLN1;2 and 1210 ± 40 μM for GLN1;3, respectively). GLN1;2 and GLN1;3 can be classified as low affinity subtypes of GS1. As for Glu, GLN1;1 and GLN1;4 again showed substantially high substrate affinities. The $K_m$ values of GLN1;1 and

**(Fig. 3.** Effects of nitrogen conditions on mRNA accumulations of GS1 isoenzymes in Arabidopsis roots. A, nitrogen deprivation for 3 days. B, ammonium excess (10 mM ammonium chloride) for 24 h. Quantitative real-time PCR analysis was performed using gene-specific primers as described under “Experimental Procedures.” The PCR products were detected and quantified as SYBR Green fluorescence. Means of independent triplicate samples and S.D. values (n = 3) are indicated.)
GLN1;4 for Glu were approximately one-fourth of those calculated for GLN1;2 and GLN1;3. Furthermore, the kinetic constants calculated for the cell culture-derived GS1 fairly matched within the range of $K_m$ and $V_{\text{max}}$ values of GLN1;2 and GLN1;3 (Table II), suggesting the cells propagated with ample ammonium supply from MS medium may contain the low affinity subtypes as major forms of GS1.

As shown in Fig. 6, the kinetics of GLN1;3 activities over Glu concentrations showed distinctive characteristics (Fig. 6C). The GLN1;3 activities followed a Michaelis-Menten saturation curve when Glu was supplied at the concentrations lower than 20 mM. GLN1;3 exhibited substantially high specific activities within this range, as indicated by a relatively high $V_{\text{max}}$ value (162.0 ± 42.0 nanokatal/mg protein) compared with those of the other isoenzymes (Table II). However, the addition of a surplus amount of Glu in the assay mixture significantly inhibited the activity of GLN1;3 (Fig. 6C). The activity was decreased ~50% of the calculated $V_{\text{max}}$ value when 80 mM of Glu was present in the reaction mixture.

**Cell Type-specific Expression of GS1 in Arabidopsis Roots**

Arabidopsis plants were transformed with fusion gene constructs of GS1 promoters and GFP. At least five independent lines from each construct were analyzed to see the identity of the expression. GFP, under the control of the GLN1;1 promoter, was abundantly accumulated in the root surface. It was detected in root tips, root hairs, and epidermis (Fig. 7, A and B).

**FIG. 4.** Fluctuation of GS1 mRNA abundance during ammonium treatment in Arabidopsis roots. Two week-old Arabidopsis plants were subjected to nitrogen starvation for 3 days prior to the treatment and then transferred to the medium without nitrogen (dashed line) or with 10 mM ammonium chloride (solid line). Total RNA was extracted from the roots at 0, 3, 6, 12, and 24 h after the treatments, and the quantitative real-time PCR analysis was performed using gene specific primers for the GS1 isoenzymes (A–D) and ubiquitin (UBQ2) (E) as a control. The PCR products were detected and quantified as SYBR Green fluorescence. Means of independent triplicate samples and S.D. values ($n = 3$) are indicated.
and F). Fluorescence of GFP was observed in the vasculature from the top to the bottom of root tissues in GLN1;2-GFP plants (Figs. 7, C–E), whereas the signal of GLN1;3-GFP was restricted in the mature part of roots (Fig. 7F) and was not detected in the root tip (Fig. 7H). GLN1;4 was the low abundant form of high affinity isoenzyme expressed under nitrogen limitation (Figs. 3 and 4). The signals of GFP in GLN1;4-GFP plants were observed in the pericycle but were restricted to the basal region of lateral root emergence (Fig. 7I).

**DISCUSSION**

Ammonium and nitrate are the predominant nitrogen sources for plants. When plants are grown under different concentrations of ammonium supply, they are capable of optimizing the rate of ammonium influx by regulating ammonium transporters (36–38). This initial regulatory step of ammonium assimilation is suggested to localize at the surface layers of roots, as indicated by the preferential accumulation of nitrogen-responsive ammonium transporters of tomato in the root hairs (39–40). Furthermore, it is suggested that *Arabidopsis* may hire a Gln-mediated negative feedback regulation for the repression of ammonium transporters (37). However, under the condition that overrides the regulatory gate of transport systems, plants are obligated to accept excessive amounts of ammonium. Under such circumstances, the activities of the downstream metabolic enzymes of nitrogen assimilation must be regulated in parallel. In the present study, we demonstrated that ammonium assimilation in *Arabidopsis* roots is regulated by finely controlled mechanisms relevant to the enzyme capacities and differential expression of the four distinct isoenzymes of GS responding to the nitrogen availabilities. It is suggested that the regulation of GS1 is significant in conjunction with the function of ammonium transporters during acclimation to the fluctuation of nitrogen environments.

Among the GLN1 genes in *Arabidopsis*, GLN1;2 was the only isoenzyme up-regulated by ammonium supply (Fig. 3), indicating that the GS1 protein accumulated there (Fig. 2, A and B) is mainly composed of GLN1;2 protein. Induction of GLN1;2 mRNA and the corresponding protein accumulation by ammonium is likely interrelated with its enzymatic properties and localization. An excess supply of ammonium can shut down the activities of ammonium transport systems (36–37) predominately localized in the root epidermis (39–40); however the ammonium may overflow this regulatory barrier and reach the inner cell layers when a surplus amount is applied to the root tissue. Under such conditions, GLN1;2 in the vasculature (Fig. 7C and D) apparently participates in Gln synthesis. The low affinity kinetics of GLN1;2 (Table II) account well for the roles postulated here. The induction of GLN1;2 mRNA by ammonium.
GLN1;1, epidermal cell; GLN1;2, root hair differentiation zone (C, F, and J); GLN1;3, and GLN1;4 were determined by expression of promoter-GFP fusion genes in transgenic Arabidopsis. A and B, GLN1;1 promoter-GFP; C–E, GLN1;2 promoter-GFP; F–H, GLN1;3 promoter-GFP; I and J, GLN1;4 promoter-GFP. Fluorescence of GFP signals in the mature part (E, H, and J) were observed by confocal laser microscopy. ep, epidermal cell; pe, pericycle cell; rh, root hair. Bar = 100 μm.

Fig. 7. Cell type-specific expression of Arabidopsis GLN1 genes in roots. Localization of GLN1;1, GLN1;2, GLN1;3, and GLN1;4 was significantly repressed by the excess supply of ammonium (Fig. 3). Fluorescence of GFP signals in the mature part (E, H, and J) were observed by confocal laser microscopy. ep, epidermal cell; pe, pericycle cell; rh, root hair. Bar = 100 μm.

Amino acid glutamine synthetase in Arabidopsis roots

GLN1;3 was the other low affinity GS1 isoenzyme expressed in Arabidopsis roots (Table II). The purified GLN1;3 enzyme showed a high capacity of Glu synthetic activity as indicated by a relatively high V_{max} value; however, the activities were significantly inhibited when Glu was supplied at the concentrations higher than 20 mM (Fig. 6C). This enzymatic characteristic of GLN1;3 may lower considerably the total GS activities in roots under the ammonium excess conditions. In addition, GLN1;3 was located in the vasculature (Fig. 7F) and was down-regulated by ammonium at the mRNA levels (Fig. 3B). These results suggest that the function of GLN1;3 is strictly controlled both at the levels of mRNA and of enzyme activities by nitrogen availabilities in Arabidopsis roots. In addition to the induction of the low capacity isoenzyme, GLN1;2, the negative regulation of GLN1;3 can substantially reduce the rate of Glu synthesis in roots under ammonium excess.

In contrast to these low affinity enzymes, GLN1;1 showed high affinities both to ammonium and glutamate with relatively low V_{max} values (Table II). The GLN1;1 mRNA was abundantly expressed in nitrogen-starved plants and was significantly repressed by the excess supply of ammonium (Fig. 3). The expression of GLN1;1 was found at the root surface, root tips, root hairs, and epidermal cells (Fig. 7A). These results strongly suggest the importance of GLN1;1 under nitrogen-limited conditions, facilitating a rapid conversion of ammonium at the cell layers that are in contact with the soil solution. During nitrogen limitation, high affinity ammonium transporters are regulated at the mRNA levels; i.e. the increase of AMT1;2 and AMT1;3 mRNA in Arabidopsis is accompanied by induction of the ammonium uptake (36–37). GLN1;1 accumulated in the root hair and epidermis may operate the Glu synthesis coupled with the action of these high affinity ammonium transporters. GLN1;4 was the other high affinity isoenzyme expressed under nitrogen-limited conditions (Fig. 3A). The GLN1;4 mRNA was less abundant than GLN1;1 and specifically localized at the pericycle cells of the basal region of lateral root emergence (Fig. 7I). These cells could have a direct contact to the soil solution due to the breakage of outer cell layers by the emergence of lateral roots. GLN1;4 may partly contribute to the Glu synthesis in these specific cell types when ammonium is undersupplied.

Taken together, both the ammonium influx and Glu synthesis are controlled by negative feedback regulation in Arabidopsis roots. The ammonium uptake was associated with the expression of high affinity ammonium transporters, induced by nitrogen limitation, and suppressed by the nitrogen supply and Glu (36–37). Unlike the case in rice (41), the induction of ammonium transporter by ammonium supply was absent in Arabidopsis (36). The present study indicated that the high affinity isoenzyme of GS1 in the root epidermis (GLN1;1) is down-regulated by ammonium; however the expression of GLN1;2 that exhibits the lowest affinity is conversely stimulated in Arabidopsis roots. In addition, the other low affinity isoenzyme, GLN1;3, which shows the highest capacity of Glu synthesis, was down-regulated by ammonium excess at the mRNA levels, and, strikingly, its enzyme activity was negatively controlled by Glu. Consequently, the overall Glu synthetic activity in Arabidopsis root is steadily controlled during ammonium treatment, which does not meet with the marked increase in GLN1;2 mRNA and the corresponding GS1 protein contents. These results suggested a unique regulatory system of ammonium assimilation in Arabidopsis, totally different from those that exist in the legumes and monocots (18–22, 41).

Acknowledgments—We thank Tetsuro Mimura (Nara Woman’s University, Japan) and Masaaki Umeda (University of Tokyo, Japan) for providing the Arabidopsis cell culture.

REFERENCES

1. Oaks, A., and Hirel, B. (1985) Annu. Rev. Plant Physiol. 36, 345–365
2. Lam, H. M., Coschigano, K. T., Oliveira, I. C., Melo-Oliveira, R., and Coruzzi, G. M. (1996) Annu. Rev. Plant Physiol. Plant Mol. Biol. 47, 569–593
3. Ireland, R. L., and Lea, P. J. (1999) in Plant Amino Acids: Biochemistry and Metabolism (Singh, B. K., ed), pp. 49–109, Marcel Dekker, New York
4. Tobin, A. K., and Yamaya, T. (2002) J. Exp. Bot. 53, 591–604
5. Gehardt, C., Olivier, J. E., Forde, B. G., Saarelainen, R., and Miflin, B. J. (1986) EMBO J. 5, 1429–1435
6. Tingey, S. V., Walker, E. L., and Coruzzi, G. M. (1987) EMBO J. 6, 1–9
7. Sutoh, T., Ogawa, M., Masumura, T., Shibata, D., Takeko, T., Tanaka, K., and Fujii, S. (1989) Plant Mol. Biol. 13, 611–614
8. Peterman, T. K., and Goodman, H. M. (1991) Mol. Gen. Genet. 224, 145–154
9. Sarukura, H., Kawai, S., Takahashi, H., Hase, T., and Sugiyama, T. (1992) Plant Cell Physiol. 33, 49–58
10. Edwards, J. M., Walker, E. L., and Coruzzi, G. M. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 5459–5463
11. Forde, B. G., Day, H. M., Turton, J. F., Shen, W.-J., Cullimore, J. V., and Coruzzi, G. M. (1996) Plant Physiol. 113, 1223–1229
12. Li, M. G., Villemur, R., Hussey, P. J., Silflow, C. D., Gantt, J. S., and Snustad, D. P. (1993) Plant Mol. Biol. 23, 401–407
13. Boche, D., Temple, S. J., and Sengupta-Gopalan, C. (1993) Plant Mol. Biol. 22, 971–983
14. Dubois, F., Brugiere, N., Sangwan, R. S., and Hirel, B. (1996) Plant Mol. Biol. 31, 803–817
15. Sakurada, N., Katayama, Y., and Yamaya, T. (2001) Physiol. Plant. 113, 400–408
16. Morey, K. R., Ortega, J. L., and Sengupta-Gopalan, C. (2002) Plant Physiol. 129, 182–193
17. Hird, B., Bouet, C., King, B., Layzell, D., Jacobs, B., and Verma, D. P. S. (1987) EMBO J. 6, 1176–1177
18. Cook, J. M., mould, R. M., Bennett, M. J., and Cummins, D. P. (1990) Plant Mol. Biol. 14, 549–560
19. Miao, G. H., Hirel, B., Marsolier, M. C., Ridge, R. W., and Verma, D. P. S. (1992) Plant Physiol. 139, 551–564
20. Sakakibara, H., Shimizu, H., Hase, T., Yamazaki, Y., Takao, T., Shimoshimizu, Y., and Sugiyama, T. (1996) J. Biol. Chem. 271, 29561–29568
21. Arabidopsis Genome Initiative (2000) Nature 408, 811–815
22. Oliveira, I. C., and Coruzzi, G. M. (1999) Plant Physiol. 121, 301–309
23. Inaba, K., Fujiwara, T., Hasada, H., Chino, M., Komeda, Y., and Naito, S. (1994) Plant Physiol. 104, 881–887
24. Mathur, J., Szabados, L., Schaad, S., Gronenberg, B., Lossow, A., Jena, Strube, E., Schell, J., Koncz, C., and Koncz-Kalan, Z. (1998) Plant J. 13, 707–716
27. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
28. Sakurai, N., Hayakawa, T., Nakamura, T., and Yamaya, T. (1996) *Planta* **200**, 306–311
29. Chiu, W. L., Niwa, Y., Zeng, W., Hirano, T., Kobayashi, H., and Sheen, J. (1996) *Curr. Biol.* **6**, 325–330
30. Koncz, C., and Schell, J. (1986) *Mol. Gen. Genet.* **204**, 383–396
31. Höfgen, R., and Willmitzer, L. (1988) *Nucleic Acids Res.* **16**, 9877
32. Clough, S. J., and Bent, A. F. (1998) *Plant J.* **16**, 735–743
33. Valverde, D., van Montagu, M., and van Lijsebettens, M. (1998) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5536–5540
34. Yoshimoto, N., Inoue, E., Saito, K., Yamaya, T., and Takahashi, H. (2003) *Plant Physiol.* **131**, 1511–1517
35. Wang, R., Okamoto, M., Xing, X., and Crawford, N. M. (2003) *Plant Physiol.* **132**, 556–567
36. Gazzarrini, S., Lejay, L., Gojon, A., Ninnemann, W. B., Frommer, W. B., and von Wirén, N. (1999) *Plant Cell* **11**, 937–947
37. Rawat, S. R., Silim, S. N., Kronzucker, H. J., Siddiqi, M. Y., and Glass, A. D. M. (1999) *Plant J.* **19**, 143–152
38. von Wirén, N., Gazzarrini, S., Gojon, A., and Frommer, W. B. (2000) *Curr. Opin. Plant Biol.* **3**, 254–261
39. Lauter, F. R., Ninnemann, O., Bucher, M., Riesmeier, J., and Frommer, W. B. (1996) *Proc. Natl. Acad. Sci. U.S.A.* **93**, 8139–8144
40. von Wirén, N., Lauter, F. R., Ninnemann, O., Gillissen, B., Walchi-Liu, P., Engels, C., Just, W., and Frommer, W. B. (2000) *Plant J.* **21**, 167–175
41. Sonoda, Y., Ikeda, A., Saiki, S., von Wirén, N., Yamaya, T., and Yamaguchi, J. (2003) *Plant Cell Physiol.* **44**, 726–734
Kinetic Properties and Ammonium-dependent Regulation of Cytosolic Isoenzymes of Glutamine Synthetase in Arabidopsis
Keiki Ishiyama, Eri Inoue, Akiko Watanabe-Takahashi, Mitsuhiro Obara, Tomoyuki Yamaya and Hideki Takahashi

J. Biol. Chem. 2004, 279:16598-16605.
doi: 10.1074/jbc.M313710200 originally published online February 2, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M313710200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 39 references, 12 of which can be accessed free at http://www.jbc.org/content/279/16/16598.full.html#ref-list-1