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# Differential Expression within the Glutamine Synthetase Gene Family of the Model Legume *Medicago truncatula*[1]

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**The glutamine synthetase (GS) gene family of *Medicago truncatula* Gaertn. contains three genes related to cytosolic GS (*MtGSa*, *MtGSb*, and *MtGSc*), although one of these (*MtGSc*) appears not to be expressed. Sequence analysis suggests that the genes are more highly conserved interspecifically rather than intraspecifically: *MtGSa* and *MtGSb* are more similar to their homologs in *Medicago sativa* and *Pisum sativum* than to each other. Studies in which gene-specific probes are used show that both *MtGSa* and *MtGSb* are induced during symbiotic root nodule development, although not coordinately. *MtGSa* is the most highly expressed GS gene in nodules but is also expressed to lower extents in a variety of other organs. *MtGSb* shows higher levels of expression in roots and the photosynthetic cotyledons of seedlings than in nodules or other organs. In roots, both genes are expressed in the absence of an exogenous nitrogen source. However the addition of nitrate leads to a short-term, 2- to 3-fold increase in the abundance of both mRNAs, and the addition of ammonium leads to a 2-fold increase in *MtGSb* mRNA. The nitrogen supply, therefore, influences the expression of the two genes in roots, but it is clearly not the major effector of their expression. In the discussion section, the expression of the GS gene family of the model legume *M. truncatula* is compared to those of other leguminous plants.**

GS (EC 6.3.1.2) is a key enzyme in the nitrogen metabolism of higher plants, catalyzing the assimilation of ammonium to form Gln. This ammonium is derived from the primary nitrogen sources of the plant (through the reduction of soil nitrate and, in the case of legumes, by the symbiotic fixation of atmospheric nitrogen), as well as from other metabolic pathways such as photorespiration, phenylpropanoid metabolism, and the catabolism of amino acids (Lea et al., 1990). These pathways occur to varying extents in different tissues and subcellular locations, which is reflected by the fact that in higher plants GS exists as a number of distinct isoenzymes located in both the cytosol and the chloroplast, which have different activities in different organs (McNally and Hirel, 1983). These multiple isoenzymes are encoded by a small family of genes that, in turn, have been shown to be differentially expressed in both a developmental- and organ-specific manner (Forde and Cullimore, 1989; McGrath and Coruzzi, 1991; Peterman and Goodman, 1991).

The GS gene family has been particularly well characterized in leguminous plants in which a crucial role is played by the cytosolic GS in the assimilation of ammonium released by nitrogen-fixing bacteria within the infected cells of the nodule. Indeed, in several legume species, the expression of one or more cytosolic GS genes has been shown to be induced during nodule development (refs. in Cullimore and Bennett, 1992). Whether this induction of GS gene expression is triggered by the presence of its substrate as a result of symbiotic nitrogen fixation remains controversial. Hirel et al. (1987) concluded that GS gene expression in soybean is directly regulated by ammonium, when either supplied externally to roots or made available as a result of nitrogen fixation in nodules. However, a similar study in bean (Cock et al., 1990) failed to find a major role for ammonium in specifically inducing GS gene expression in either roots or nodules.

The role of GS gene expression is of particular interest in the forage legume alfalfa (*Medicago sativa*), because there is evidence that the levels of GS activity within the nodules may have an effect on nodule development, the rate of nitrogen fixation, and the eventual productivity of the plant (Knight and Langston-Unkefer, 1988). Studies of GS gene expression have already been initiated in alfalfa (Tischer et al., 1986; Dunn et al., 1988). However, molecular and genetic studies are complicated in this species by the fact that *M. sativa* has a large genome and is tetraploid and out-breeding. Although *Arabidopsis thaliana* has proved its value as a model higher plant for a plenitude of molecular studies, including the characterization of the GS multigene family (Peterman and Goodman, 1991), it is unfortunately not possible to use this plant to address the role of such genes within the nodule. We have chosen *Medicago truncatula* Gaertn. as the model legume for studying the symbiotic relationship between *Medicago* species and the nitrogen-fixing bacterium *Rhizobium meliloti* (Barker et al., 1990). Although *M. truncatula* is a close relative to *M. sativa*, it offers the advantages of being diploid and self-fertilizing and of having a relatively small genome. It is also amenable to genetic manipulation, being readily transformed via *Agrobacterium tumefaciens* and regenerated by somatic embryogenesis (Thomas et al., 1992).

Here we report the characterization of the cytosolic GS

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Abbreviations: GS, glutamine synthetase; NAR nodulation in the absence of *Rhizobium*; SSC, standard sodium citrate.

gene family of *M. truncatula*. Using gene-specific probes, we have investigated the expression of different members of the gene family within the different organs of the plant and particularly within the nodule. Also, we have addressed the question as to whether GS gene expression is regulated by the plant's nitrogen supply.

## MATERIALS AND METHODS

### Plant Material

For the analysis of different organs, seeds of *Medicago truncatula* Gaertn. cv Jemalong (A28–28) were surface sterilized and then germinated on solidified Fahraeus medium in the dark at 13°C. The sprouting seeds were either dissected after 2 d of growth or transferred aseptically to agar slants of Fahraeus medium containing 2 mM $KNO_3$ and propagated for 2 to 3 weeks under a 16-h light photoperiod. To investigate the effects of the nitrogen supply to the roots, seedlings were grown on agar slants of Fahraeus medium containing either no nitrogen source, 2 mM $KNO_3$, 2 mM $NH_4Cl$, or 2 mM $NH_4NO_3$. For studying nodule development and the short-term effects of nitrogen starvation and readdition, plants of *M. truncatula* were grown in aeroponic conditions for 2 to 4 weeks on a medium containing 5 mM $NH_4NO_3$ at 20°C with an RH of 75% and a 14-h light photoperiod (Lullien et al., 1987). For nodule induction, the growth medium was removed and replaced with fresh medium lacking a nitrogen source 2 d before inoculation with the wild-type *Rhizobium meliloti* strain 2011. $Fix^-$ nodules of *Medicago sativa* L. cv Gemini were likewise induced following inoculation with either the *nifA* mutant *R. meliloti* strain 1354 (Szeto et al., 1984) or the *exoB* mutant strain EJ355 (Finan et al., 1985). Plants having an NAR phenotype (line *nslD*) were grown under the same conditions without inoculation (Truchet et al., 1989). In our investigations of the effects of the nitrogen supply, plants were starved of nitrogen for 6 d before the readdition of a nitrogen source to the medium [10 mM $KNO_3$ or 5 mM $(NH_4)_2SO_4$].

### Isolation of Genomic Clones λpGSa1, λpGSb1, and λpGSc1

Clones encoding GS were isolated from a genomic library of *M. truncatula* cv Jemalong by the methods of Gallusci et al. (1991), using a partial cDNA probe (750-bp *Pst*I fragment) encoding the GS γ polypeptide of *Phaseolus vulgaris* (Cullimore et al., 1984). Hybridization was carried out at 37°C in 50% formamide, with subsequent washing in 2× SSC, 0.1% SDS at 60°C (1× SSC contains 0.15 M NaCl and 0.015 M trisodium citrate at pH 7.0).

### DNA Sequencing

Restriction fragments containing the *MtGSa*, *MtGSb*, and *MtGSc* genes were subcloned into pUC19 and BlueScript (Stratagene) vectors using techniques essentially as described by Maniatis et al. (1982). Dideoxy sequencing using double-stranded plasmid DNA was performed according to the method of Chen and Seeburg (1985) using the Sequenase kit (United States Biochemical). Sequence alignments were made over 153 bp of both the coding sequence and the contiguous 3′ noncoding sequence using the Bestfit Program (for pairwise comparisons) or the Clustal Program (for multiple alignments) (Devereux et al., 1984). Default parameters were used, and homologies were calculated as a percentage of identical alignments.

### Northern Analyses

Total RNA was isolated from various organs by phenol/$CHCl_3$ extraction using a high pH buffer (Jackson and Larkins, 1976). Samples of 10 μg were separated in 1% agarose-formaldehyde gels, together with 2-fold dilution standards of one of the samples. These dilutions were later used to quantify the relative hybridization signals. Following electrophoresis, gels were stained with ethidium bromide to check the loadings. After direct transfer to GeneScreen Membrane (NEN), blots were hybridized according to the GeneScreen protocol at 37°C in the presence of 50% formamide and 10% dextran sulfate with [$\alpha$-$^{32}$P]dCTP-labeled probes ($1 \times 10^9$ cpm/μg) prepared by random oligo-priming of gel-purified DNA fragments (Feinberg and Vogelstein, 1983). Thus, for *MtGSa*, we used a 280-bp *Ava*II/*Hin*dIII fragment that is located 29 bp downstream from the translational stop, for *MtGSb*, we used a 400-bp *Nsi*I/*Eco*RI fragment located 36 bp downstream from the translational stop, and for *MtGSc*, we used a 280-bp *Sty*I/*Hin*cII fragment located 3 bp downstream from the translational stop. These three probes can be regarded as gene specific in DNA:RNA hybridizations because there is no cross-hybridization between in vitro transcribed [$\alpha$-$^{32}$P]UTP-labeled RNA probes derived from each fragment and either of the other two genes. All blots were washed at 60°C in 2× SSC, 0.1% SDS and subjected to autoradiography. The relative abundances of the GS mRNAs in the different samples were estimated by comparison to the signals from the dilution samples. The stripping of probes from blots was performed according to the specifications of the GeneScreen manufacturer.

### Southern Hybridization Analysis

DNA was isolated from leaves of *M. truncatula* (Dellaporta et al., 1983) and from the GS phasmids (Maniatis et al., 1982). Samples of 3 μg of plant DNA or 500 pg of phasmid DNA were digested with the restriction enzymes *Eco*RI and *Hin*dIII, and the fragments were separated on 1% agarose gels. Blotting and hybridization were carried out as described for northern analysis using the same probes relating to the *MtGSa*, *MtGSb*, and *MtGSc* genes. In addition, the blots were hybridized with probes prepared to the 3′ *Bam*HI fragment of the *P. vulgaris* chloroplast GS cDNA (Lightfoot et al., 1988) and to the 3′ *Pst*I fragment of a cytosolic GS cDNA (Cullimore et al., 1984).

## RESULTS

### Isolation of Genomic Clones Encoding Cytosolic GS of *M. truncatula*

A genomic library of *M. truncatula* DNA was screened for GS clones by hybridization with a heterologous probe pre-

pared from a cDNA encoding the last 133 amino acids of a cytosolic GS polypeptide of *P. vulgaris* (Cullimore et al., 1984). A number of clones were isolated that related to only three different types, represented by the phasmid clones λpGSa1, λpGSb1, and λpGSc1. The restriction maps and the position of the GS genes in these three clones are shown in Figure 1. The different restriction maps suggest that the three clones relate to different genes that were designated *MtGSa*, *MtGSb*, and *MtGSc*, respectively.

To distinguish clearly between the expression of these different members of the GS gene family, it was necessary to define gene-specific probes. Previous analyses of GS gene families from other leguminous species have shown that, although the coding region remains well conserved, the 3′ noncoding sequences are often very divergent (Forde and Cullimore, 1989; Peterman and Goodman, 1991). Sequencing

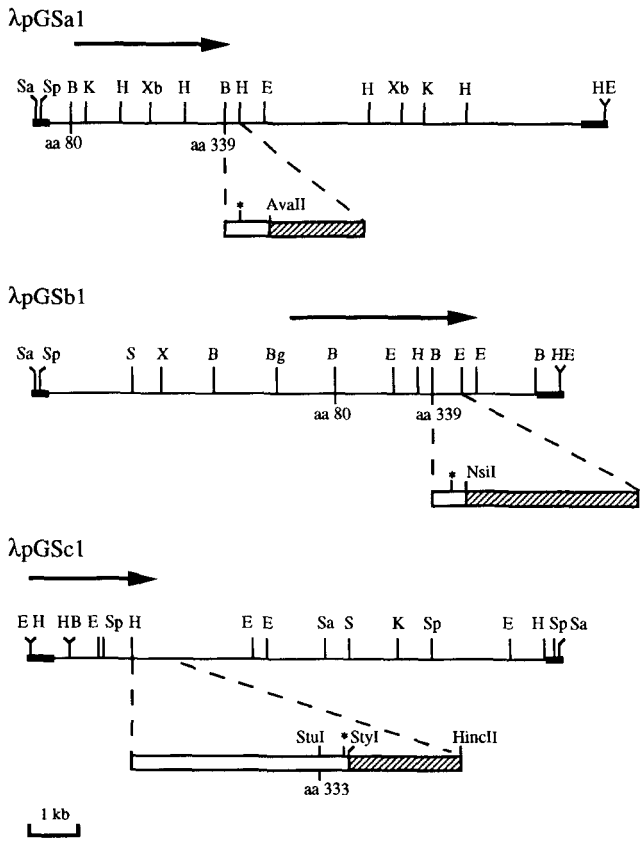


**Figure 1.** Partial restriction maps for the genomic clones containing *M. truncatula* GS genes *MtGSa*, *MtGSb*, and *MtGSc*. The positions and orientations of the three genes are shown by arrows relative to the bordering sites of the phasmid-cloning vector pGY97 (Vincze and Kiss, 1990), thick lines representing pBR322 DNA sequences. The correspondence between amino acid (aa) positions and certain restriction sites within the coding sequences are given underneath. The boxed regions below the maps (drawn 8 times larger than scale) were completely sequenced, and the indicated restriction sites were unique within these fragments. The shaded regions of the boxes located 3′ to the translational stop codons (marked with asterisks) delimit the fragments used for gene-specific probes. Sa, *Sal*I; Sp, *Sph*I; B, BamHI; K, *Kpn*I; H, *Hin*dIII; Xb, *Xba*I; E, *Eco*RI.

```
          1                                                  50
MtGSa GGTGTGGCAA ACCGTGGTGC ATCTGTTAGG GTTGGAAGAG ACACAGAAAA
MtGSb GGTGTTGCAA ACCGTGGTGC GTCGATTAGA GTTGGAAGGG ACACAGAGAA
MtGSc GGTGTTGCGA ACCGTGGTGC TTCTGTTAGG GTAGGAAGGG AGACTGAGAA
      ***** ** * **********  **  ****  ** ***** * * ** ** **

          51                                                 100
MtGSa AGATGGCAAA GGTTACTTTG AGGATAGAAG GCCTTCTTCT AATATGGATC
MtGSb AGCAGGAAAA GGTTATTTCG AGGACAGGAG GCCATCATCT AACATGGATC
MtGSc AGCAGGGAAG GGATATTTTG AGGACAGAAG GCCTGGATCT AACATGAATC
      **  ** **  ** ** ** * **** ** ** ***    *** ** *** ***

          101                                                150
MtGSa CCTATGTGGT TACTTCCATG ATTGCTGAAA CTACCCTTCT ATGGAAACCA
MtGSb CATATGTTGT TACTTCCATG ATTGCAGACA CCACCATTCT CTGGAAACCA
MtGSc CGTATGTGGT CACATCCATG ATAGCAGAGA CAACCATCCT CTGGAAACCA
      * ***** **  ** ****** ** ** ** * * *** * **  *********

          151                                                200
MtGSa TGAAGTCACA ACCACTTGTC TTTGGATTTG GACCACATCA CATTATGTGT
MtGSb TAAGCCACCA CGCTACTTCT GCTATAAAAC ACACACATGC ATTGTAGTCT
MtGSc TGAACAACCT TGGAATTACC AGCACATATT AGTACTAAGT TGTGATATTT
      * *    *       *       *         *     *   * *

          201                                                250
MtGSa TCAATAAATA GACTAGTTTA TATCTATTAT CATTATCATT ATGCAAAGTG
MtGSb TTCAAAGTCA TTGTTGATTC CGCATTAGAA TTTGGTCATT GGTTTTTCTA
MtGSc TGCTTTTATT AATTTGCTTG TTTTTCCTTA GGACTTTTTA GTCGCATGAT
      *           * * **     *         * *

          251                                                300
MtGSa TTGCTTCATT AGTAGTAATT GTCCAAGTGG TCTTTAAGCA CAAAGCATTT
MtGSb GGATTTGATT TGTGTTATTG TTATGGTTCA CACTTTGTTT GTTTGGATCT
MtGSc GTTTAATTAT GGGCTTGTTT GACTTTGGTT TCCATTGCTT TATGAGAGTC
            * *  * *              *          *

          301
MtGSa AGTCCA
MtGSb GAGTCC
MtGSc ACTTTG
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**Figure 2.** Nucleic acid sequences of the last exon and adjacent 3′ noncoding sequence of the three *M. truncatula* GS genes *MtGSa*, *MtGSb*, and *MtGSc*. The positions of the stop codons are boxed, and stars represent positions of identity among the three genes.

near the 3′ ends revealed that all three *M. truncatula* GS genes contain an intron at the same position within the coding sequence as the last intron described in a *M. sativa* GS gene (Tischer et al., 1986). Therefore, we sequenced downstream of this intron, through the last exon and 3′ noncoding region for each of the three genes (Fig. 2). A comparison of these partial sequences clearly shows a high conservation of the coding sequences in contrast to the 3′ noncoding sequences (Fig. 2). This conclusion was reinforced by Bestfit pairwise comparisons of the coding and noncoding sequences of the three genes (summarized in the first two columns of Table I). Similar pairwise comparisons were made (Table I) against the corresponding regions of GS sequences described for alfalfa and pea. This clearly showed *MtGSa* to be the homolog of the nodule-specific cDNA described in alfalfa (Dunn et al., 1988) with 97% homology over the coding region and 95% in the 3′ noncoding sequences. It also had a relatively high degree of homology (89 and 65%, respectively) with GS341

**Table I.** *Sequence similarities between legume GS genes*

The values show the percentage identities of the coding sequences (upper, bold values) and 3′ noncoding sequences (lower values) among GS genes *MtGSa*, *MtGSb*, and *MtGSc* from *M. truncatula* and GS genes from *M. sativa*, here referred to as *MsN* (Dunn et al., 1988) and *MsT* (Tischer et al., 1986), and from *P. sativum*, here referred to as *Ps341* (Tingey et al., 1987) and *Ps299* (Tingey et al., 1988). Boxed values are shown for the homologs identified to *MtGSa* (first row) and *MtGSb* (second row).

| | *M. truncatula* | | *M. sativa* | | *P. sativum* | |
|---|---|---|---|---|---|---|
| | *GSb* | *GSc* | *MsN* | *MsT* | *Ps341* | *Ps299* |
| *GSa* | **84** | **80** | **97** | **84** | **89** | **81** |
| | 45 | 37 | 95 | 42 | 65 | 33 |
| *GSb* | | **83** | **84** | **99** | **84** | **89** |
| | | 40 | 45 | 82 | 47 | 71 |
| *GSc* | | | **81** | **83** | **80** | **85** |
| | | | 32 | 46 | 41 | 48 |

from *Pisum sativum* (Tingey et al., 1987). *MtGSb* appears to be the homolog of a second GS gene described in *M. sativa* that was found to be amplified in a cell culture line selected for its resistance to the herbicide L-phosphinothricin, a competitive inhibitor of GS (Tischer et al., 1986). It also shows strong homology with GS299, another cytosolic GS gene described for pea (Tingey et al., 1988).

To investigate whether there are additional cytosolic GS genes in the *M. truncatula* genome and to investigate the specificity of probes for each of the three genes, Southern hybridization was performed to *Eco*RI digests of *M. truncatula* DNA (Fig. 3). The *P. vulgaris* cytosolic GS probe, used to isolate the three genes, hybridized to three genomic fragments of 1.4, 3.2, and 7.0 kb. The former two fragments correspond in size to the 3′ *Eco*RI fragments of *MtGSb* and *MtGSc*, respectively, whereas the 3′ fragment of *MtGSa* should be at least 4.2 kb from the restriction map. That the three genomic fragments do indeed relate to the three cloned genes was shown by hybridization with probe fragments taken from the 3′ noncoding region of each gene. Each probe fragment hybridized only to its expected genomic fragment, thus showing that the 3′ noncoding sequence probes are gene specific. A probe containing coding sequences from the 3′ end of *MtGSb* hybridized to all three fragments and not to any others (data not shown), thus reinforcing the conclusion that there are only three GS genes encoding cytosolic GS in the *M. truncatula* genome. In addition, results of Southern hybridization with the 3′ region of the chloroplastic GS cDNA of *P. vulgaris* (Lightfoot et al., 1988) suggest that there is at least one extra gene encoding a plastid-GS counterpart.

## Differential Organ-Specific Expression of the GS Gene Family in *M. truncatula*

The expression of the three genes *MtGSa*, *MtGSb*, and *MtGSc* was examined in various organs of *M. truncatula* by northern analysis. Total RNA was isolated from roots, leaves, stems, petioles, flowers, and dry seeds, as well as from mature nodules (28 d postinoculation) and germinating seeds. In the latter two situations, large amounts of Gln are synthesized, and GS activity has been shown to increase (Walker and Coruzzi, 1989; Swarup et al., 1990; McGrath and Coruzzi, 1991). Gene-specific probes were prepared from DNA fragments encompassing the divergent 3′ noncoding sequences of the three genes (Fig. 1 and "Materials and Methods"). Both the *MtGSa*- and *MtGSb*-specific probes hybridized to RNA species of approximately 1.4 kb. However, the relative abundance of these two mRNAs within the different organs of the plant varied markedly (Fig. 4) Thus, mRNA corresponding to *MtGSa* was found to be most abundant in nodules. However, unlike its alfalfa homolog, which is reported to be nodule specific (Dunn et al., 1988), *MtGSa* is also highly expressed in stems and at lower levels in nearly all other organs of the plant, including roots. In contrast, high levels of mRNA corresponding to *MtGSb* were detected in several organs of the plant, most notably in roots and green cotyledons, and to a lesser extent in nodules, stems, petioles, and flowers. Most striking is the dramatic increase in the levels of *MtGSb* mRNA between seed cotyledons (dissected 2 d after sowing) and green cotyledons that were harvested 15 d later. Surprisingly, no mRNA species complementary to the *MtGSc*-specific probe could be detected using the same conditions of hybridization, even after longer exposure of the autoradiograph (data not shown).

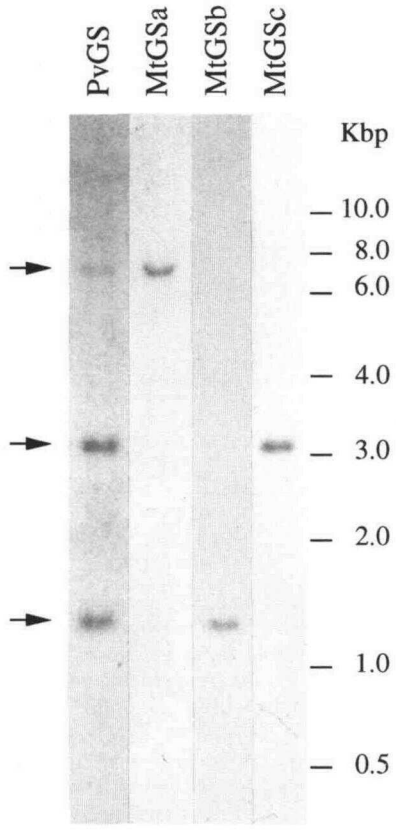


**Figure 3.** Southern hybridization to *Eco*RI digests of *M. truncatula* DNA using probes from a *P. vulgaris* cytosolic GS gene (*PvGS*) and specific 3′ noncoding sequences from *MtGSa*, *MtGSb* and *MtGSc*.

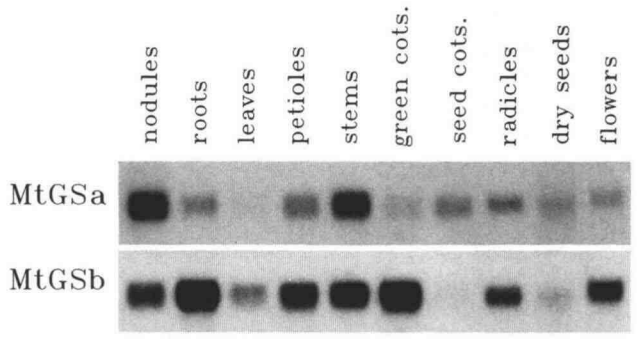


**Figure 4.** Northern analysis of differential organ-specific expression of genes *MtGSa* and *MtGSb* in *M. truncatula*. Total RNA was isolated from nodules, roots, leaves, petioles, stems, green cotyledons (cots.), cotyledons, and radicles of germinating seeds, dry seeds, and flowers. The upper part of the figure shows the autoradiograph resulting from hybridization with the *MtGSa*-specific probe. The lower part of the figure shows the same blot after stripping and subsequent hybridization with the *MtGSb*-specific probe.

### Induction of *MtGSa* and *MtGSb* Gene Expression in Developing Nodules

The observation that high levels of *MtGSa*- and *MtGSb*-specific mRNA were present in mature nodules led us to examine the expression of these two genes over a time course of nodule development. RNA was isolated from nodules harvested at various times after inoculation with *R. meliloti* and used in northern hybridization with the *MtGSa*- and *MtGSb*-specific probes. Although nodules were large enough to be picked cleanly from the root system from d 6 onward, we were obliged, for technical reasons, to excise nodule "bumps" with an adjoining root section at d 5 after inoculation. However, we estimate that in this sample the contamination of the developing nodule RNA with root RNA is less than 10%.

The abundance of mRNA of both genes was dramatically increased during nodulation from very low basal levels at d 5 (Fig. 5A). However, clear differences were observed with respect to the kinetics of accumulation of their respective RNAs. *MtGSa*-specific RNA accumulated very rapidly from barely detectable levels at d 5 to maximal levels at d 7, the high level of RNA being maintained for up to 28 d after inoculation. In contrast, *MtGSb* mRNA was present at slightly higher levels in emerging nodules (but much lower than uninoculated roots) and accumulated progressively in the developing nodule up to at least d 28. These results were confirmed in other experiments.

In soybean, it has been reported that induction of GS gene expression in nodules is dependent on ammonium derived from nitrogen fixation (Hirel et al., 1987). However, preliminary work in alfalfa by Dunn et al. (1988) has suggested that the nodule-specific GS gene is induced in certain Fix$^-$ nodules produced by mutant *R. meliloti* and, thus, that the expression of this gene is independent of nitrogen fixation. The high degree of homology existing between the 3′ noncoding sequences of *MtGSa* and *MtGSb* and their *M. sativa* homologs (Table I) allowed us to investigate these putative differences further by using the gene-specific probes of *M. truncatula* in heterologous hybridizations to RNA extracted from Fix$^-$ *M. sativa* nodules. The levels of the two GS mRNAs were thus measured in Fix$^-$ alfalfa nodules following inoculation with either *nifA* or *exoB* mutants of *R. meliloti*, which form nodules with and without intracellular bacteria, respectively. In addition, the GS mRNA levels were measured in spontaneous nodules arising in the complete absence of *Rhizobium* due to the NAR plant phenotype (Truchet et al., 1989). The results of these heterologous hybridizations are shown in Figure 5B. Although homologs to *MtGSa* and *MtGSb* were expressed at high levels in *nifA* mutant nodules, both genes are poorly expressed in both *exoB* and NAR nodules with levels of the two mRNAs being similar to the basal levels observed in emerging *M. truncatula* nodules.

### Regulation of GS Gene Expression in Response to the Nitrogen Supply

The discrepancy between our observation that homologs of *MtGSa* and *MtGSb* are induced in *M. sativa* nodules in the absence of nitrogen fixation and that of Hirel et al. (1987), who reported that nitrogen fixation is required for the induction of GS genes in soybean, prompted us to investigate whether there is also a difference between the two species with regard to the regulation of GS gene expression in roots. In soybean, expression of GS genes is induced by addition of ammonium, but not nitrate, to nitrogen-starved roots (Hirel et al., 1987).

Our first approach was to assay the level of mRNA corresponding to both *MtGSa* and *MtGSb* in the roots of *M. truncatula* plants that had been grown using different nitrogen regimens. Total RNA was isolated from the roots of seedlings that had been grown on either nitrogen-deficient or nitrate-, ammonium-, or ammonium nitrate-containing medium, both 10 and 20 d after germination. Hybridization with gene-specific probes for *MtGSa* and *MtGSb* was then performed. The resulting autoradiograph in Figure 6A clearly shows that the nitrogen supply does not greatly affect the expression of either *MtGSa* or *MtGSb*, because both genes are

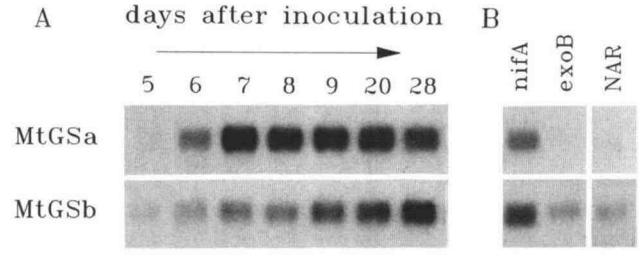


**Figure 5.** Northern analysis of *MtGSa* and *MtGSb* expression during nodule development in *M. truncatula* and the expression of their homologous genes in nonfixing *M. sativa* nodules. Total RNA was isolated from *M. truncatula* nodules harvested 5 to 28 d after inoculation with *R. meliloti* (A) and Fix$^-$ *M. sativa* nodules (28 d old), formed either as a result of inoculation with *nifA* or *exoB* bacterial mutants or spontaneously generated on NAR plants (B). The upper and lower parts of the figure show the results of hybridization with *MtGSa*- and *MtGSb*-specific probes, respectively.

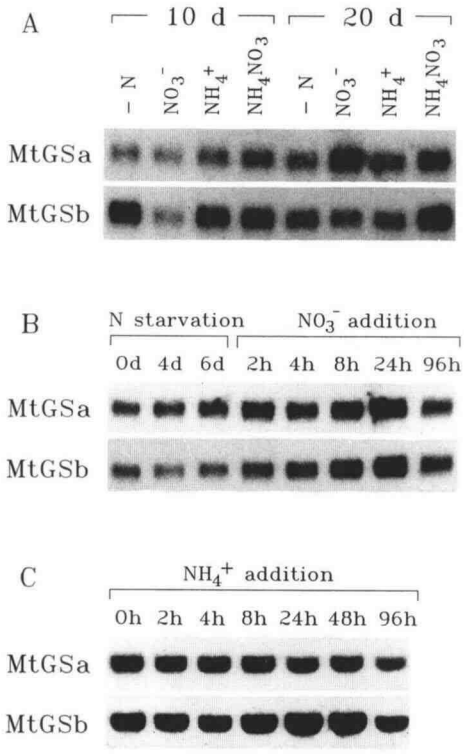


**Figure 6.** Effect of exogenously supplied nitrogen on the expression of *MtGSa* and *MtGSb* in roots. *MtGSa*- and *MtGSb*-specific probes were hybridized to blots of total RNA isolated from roots of *M. truncatula* plants grown under the following conditions: A, Seedling plants grown for 10 or 20 d on solid medium containing either no nitrogen source (−N), 2 mM $KNO_3$, 2 mM $NH_4Cl$, or 2 mM $NH_4NO_3$; B, plants (2–4 weeks old) grown under aeroponic conditions on a medium containing 5 mM $NH_4NO_3$ were subsequently starved of nitrogen during a 6-d period (sampled at 0, 4, and 6 d), before the readdition of 10 mM $KNO_3$ to the medium (sampled 2, 4, 8, 24, and 96 h after $NO_3^-$ addition); C, plants that had been grown under aeroponic conditions were starved of nitrogen during a 6-d period and then exposed to a medium containing 5 mM $(NH_4)_2SO_4$ (sampled 2, 4, 8, 24, 48, and 96 h after $NH_4^+$ addition). For experiment A, the roots from three plants were harvested for each sample, and for B and C, two whole root systems were harvested.

expressed at relatively high levels irrespective of the nitrogen source and, notably, even in the absence of mineral nitrogen. However, a small increase (2- to 3-fold) in the levels of both mRNAs was observed between 10 and 20 d for plants grown in the presence of nitrate.

It may be argued that plants grown without nitrogen in the medium were still receiving a supply of nitrogen from the mobilization of protein reserves within the seed. This is unlikely because 20-d-old seedlings showed clear signs of nitrogen starvation: their leaves were chlorotic and there was a marked reduction in the growth of their aerial parts compared to plants grown on media containing either nitrate or ammonium nitrate. Furthermore, an extended growth period of 30 d resulted in the death of plants growing on nitrogen-deficient medium, as well as of plants grown on ammonium. In the latter case, this was not due to acidification of the growth medium because the pH was not significantly lower than that of the other growth media. In conclusion, at 20 d postgermination when plants were clearly being subjected to differences in their nitrogen supply, the level of mRNAs for both *MtGSa* and *MtGSb* were not markedly different among the four regimens.

Although it is possible to conclude that, for plants grown continuously using different nitrogen regimens, the nitrogen supply has little overall effect on the expression of *MtGSa* and *MtGSb*, the nature of this first experiment would not allow the detection of rapid changes in gene expression in response to sudden changes in the available nitrogen. Because of the findings by Hirel et al. (1987), who reported an increase in GS gene expression in soybean roots only 2 h after the addition of ammonium, we decided to perform short-term nitrogen starvation and readdition experiments. The use of plants grown under aeroponic conditions allowed a greater flexibility in the manipulation of the nitrogen supply to the roots. The results in Figure 6B clearly show that the expression of neither *MtGSa* nor *MtGSb* is altered in response to the withdrawal of the nitrogen source on which the plants were grown, even after 6 d of starvation. This is in agreement with the results of the nitrogen-deficient regimen used in the previous test-tube experiment. However, the addition of nitrate led to a 2- to 3-fold increase in the levels of *MtGSa*- and *MtGSb*-specific mRNA during the first 24 h, which returned to basal levels by 96 h.

Using the same plants following regeneration of the root systems in the presence of a nitrogen source, we again imposed a 6-d starvation period before adding back ammonium as the sole nitrogen source. In the case of *MtGSa*, no change in mRNA levels is detectable, whereas for *MtGSb*, there is an approximate 2-fold increase in the abundance of the respective mRNA between 4 and 24 h after the addition of ammonium. The level of this message is maintained at 48 h, before again returning to basal levels by 96 h.

In all these experiments on roots, the steady-state level of *MtGSb* mRNA is approximately 10-fold higher than that of *MtGSa* RNA; in Figure 6 the exposure of the autoradiographs has been adjusted to show signals of similar intensities.

## DISCUSSION

The key position that GS occupies in the plant's nitrogen metabolism is reflected by the numerous molecular studies of this enzyme. Many of these studies have involved leguminous plants in which cytosolic GS plays a critical role in the assimilation of symbiotically fixed nitrogen. The GS genes have been best characterized in bean (Forde and Cullimore, 1989) and pea (Tingey et al., 1988; Walker and Coruzzi, 1989), both of which contain three expressed genes encoding cytosolic GS polypeptides and an additional single gene encoding a polypeptide that is imported into the chloroplasts. There are, however, differences between these two GS gene families: for example, *P. vulgaris* contains an additional GS sequence (*gln-ϵ*) that appears not to be expressed (Forde et al., 1989), and in *P. sativum*, two of the cytosolic GS genes have virtually identical sequences (Walker and Coruzzi, 1989).

In this paper we report the isolation of distinct genomic

clones related to three cytosolic GS genes (*MtGSa*, *MtGSb*, and *MtGSc*) of the model legume *M. truncatula*. In addition, we have identified by heterologous hybridization the existence of at least one other gene encoding a chloroplastic GS. We conclude that all members of the cytosolic GS gene family have been identified. If additional genes do exist, they either must be sufficiently unrelated so that they do not cross-hybridize in genomic Southern blots or are so similar that they cannot be distinguished by digestion with several different restriction enzymes. Thus, the GS gene family of *M. truncatula*, like that recently described for the model higher plant *Arabidopsis* (Peterman and Goodman, 1991), appears to be as complex as the gene families of bean and pea, despite the relatively small genomes of the two model species.

Homologs to two of the *M. truncatula* genes have been identified in both alfalfa and pea. Thus, *MtGSa* is the homolog of the nodule-specific gene described for alfalfa (Dunn et al., 1988) and of GS341 from pea (Tingey et al., 1987), whereas *MtGSb* is homologous to a second GS gene from alfalfa (Tischer et al., 1986) and to GS299, another cytosolic gene from pea (Tingey et al., 1988). The observation that these GS genes are more highly conserved interspecifically than within the gene family of *M. truncatula* itself suggests that the process of duplication and divergence within the GS gene family must have occurred before speciation of the legumes. However, this conservation of sequences is restricted to closely related tribes in the Papilionoideae, such as the Trifolieae (includes *Medicago*) and the Vicieae (includes *Pisum*) (Polhill, 1981), because homologs could not be identified by comparison with GS sequences from *P. vulgaris* (a member of the more distantly related Phaseoleae).

Our analysis of the expression of the GS gene family in *M. truncatula* revealed a complex pattern of differential organ-specific expression. *MtGSa* is most highly expressed in nodules but is also expressed at high levels in stems, and its mRNA is detectable in most other organs except for leaves. On the other hand, *MtGSb* shows highest expression in roots and green cotyledons and was expressed at moderately high levels in a number of other organs, including nodules. Surprisingly, no mRNA corresponding to *MtGSc* could be detected in any of the organs examined, suggesting that this gene is not expressed, as is the case for the fifth GS sequence (*gln-ϵ*) from *P. vulgaris* (Forde et al., 1989). However, it is possible that our techniques are not sufficiently sensitive to detect a low level of *MtGSc*-specific RNA or, alternatively, that this gene is expressed only at certain stages of development, which have not been examined here. We have shown that the expression of both *MtGSa* and *MtGSb* is induced during nodule development, albeit noncoordinately. RNA specific to *MtGSa* accumulates rapidly from barely detectable levels at d 5 after inoculation with *R. meliloti* to steady-state levels at d 7, whereas *MtGSb*-specific mRNA accumulated progressively during nodule development from higher basal levels. It is interesting that analysis of the same experiment has shown that the appearance of *MtGSa*-specific mRNA at d 6 after inoculation and the attainment of steady-state levels by d 7 parallel the kinetics of expression of one of the *M. truncatula* genes (*Mtlb1*) encoding the late nodulin leghemoglobin (Gallusci et al., 1991). These results suggest that these two genes may respond to the same physiological signals in nodules.

The nodule-enhanced expression of *MtGSa* contrasts with the nodule-specific expression described for its alfalfa homolog (Dunn et al., 1988). This discrepancy may be due to differences in the sensitivity of mRNA detection between the two studies, as well as the fact that in the earlier study of *M. sativa* only a limited number of organ types (i.e. leaves, roots, and nodules) were examined. Parallels may be drawn between *MtGSa* and its homolog in pea, GS341, because both of these genes are expressed in several organs but show an induction of expression during nodule development (Tingey et al., 1987; Brears et al., 1991). However, it is difficult to draw an analogy between the pattern of expression of these two homologs in a second developmental context in which large amounts of Gln are synthesized, that of the mobilization of the nitrogen reserves in the cotyledons of germinating seeds. Whereas GS341 shows a marked increase in expression in the cotyledons of germinating peas (Walker and Coruzzi, 1989), the level of *MtGSa*-specific mRNA is relatively low in the cotyledons of *M. truncatula* at both early and late stages of germination. *MtGSb* mRNA, on the other hand, is barely detectable in germinating seed cotyledons of *M. truncatula*, but unlike its homolog GS299 from pea, which shows no expression in the cotyledons of germinating pea seeds (Walker and Coruzzi, 1989), we observed a dramatic accumulation of *MtGSb*-specific RNA in the green fleshy cotyledons of *M. truncatula* seedlings. These differences in the patterns of expression of the two pairs of GS homologs may reflect the different germination strategies used by pea and alfalfa. Thus, whereas pea exhibits hypogeal germination with the cotyledons remaining underground, the germination of alfalfa is epigeal with the cotyledons developing above ground into a fully photosynthetic organ. The consequence of these different types of germination has already been reported by Edwards et al. (1990), who observed high levels of expression from the promoter of the pea chloroplast GS gene in transgenic tobacco, which shows epigeal germination, whereas this gene is normally expressed at very low levels in the nonphotosynthetic (hypogeal) cotyledons of pea.

Previous studies have addressed the question regarding the role of the plant's nitrogen supply in regulating the expression of GS gene expression. Hirel et al. (1987) concluded that genes encoding cytosolic GS in soybean are directly induced by ammonium, either supplied externally or made available as a result of nitrogen fixation. Furthermore, they have shown that regulation is substrate specific because nitrate has no inducing effect. These observations have recently been confirmed by reporter gene studies that show that the expression from the soybean GS gene promoter is induced by ammonium only in transgenic *Lotus corniculatus*, a legume, and not in transgenic tobacco (Miao et al., 1991). In contrast, Cock et al. (1990) failed to find an effect of ammonium on specifically inducing the expression of GS genes in either the roots or nodules of bean. Thus, whether GS gene expression is substrate inducible or not may depend on the species in question.

Our own study has led to the conclusion that the plant's nitrogen supply is not the key effector of GS gene expression in *M. truncatula*, but it nevertheless clearly has some effect.

In roots, the expression of *MtGSa* and *MtGSb* is largely independent of the nitrogen source, and the two genes are highly expressed even in the absence of exogenous nitrogen. However, minor changes in gene expression were observed in response to both ammonium and nitrate: an approximate 2-fold increase in the levels of both mRNAs was observed within 24 h after the addition of nitrate to nitrogen-starved roots, and a similar accumulation of mRNA specific to *MtGSb* was observed following the addition of ammonium. In alfalfa, the homologs to *MtGSa* and *MtGSb* were highly induced in $Fix^-$ nodules containing bacteroids defective for nitrogen fixation. This confirms preliminary results reported by Dunn et al. (1988) and reinforces our conclusion that the nitrogen supply is not the principal effector of GS gene expression in *Medicago* species. It is interesting that the two GS genes were poorly expressed both in alfalfa nodules formed with $Exo^-$ *R. meliloti* mutants in which bacteria are not present intracellularly and also in the spontaneously produced nodules (NAR phenotype), where there are no bacteria at all. These results concur with the suggestion that other signals, possibly associated with the development of the bacteroids (Dunn et al., 1988) or the differentiation of densely cytoplasmic host cells (Norris et al., 1988), are required for the induction of GS gene expression in nodules. Furthermore, the lack of induction of leghemoglobin genes both in nodules induced with the $exoB^-$ mutant (Dunn et al., 1988; Norris et al., 1988) and in NAR nodules (Truchet et al., 1989) suggests that the controls governing the expression of the GS genes in nodules may be common to late nodulins.

In conclusion, the cytosolic GS genes of *M. truncatula*, like those of several other legumes studied to date, are represented by a small gene family that is differentially expressed. The availability of a transformation and regeneration system for *M. truncatula* (Thomas et al., 1992) should now allow genetic engineering to be used to investigate further both the regulation and the roles of each of these genes in the nitrogen metabolism of the plant, particularly in relation to the regulation of nitrogen fixation in the nodules and the productivity of the plant.

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