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The 3' untranslated region of a soybean cytosolic glutamine synthetase (GS1) affects transcript stability and protein accumulation in transgenic alfalfa

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Summary

Higher plants assimilate nitrogen in the form of ammonia through the concerted activity of glutamine synthetase (GS) and glutamate synthase (GOGAT). The GS enzyme is either located in the cytoplasm (GS₁) or in the chloroplast (GS₂). Glutamine synthetase 1 is regulated in different plants at the transcriptional level and there are some reports of regulation at the level of protein stability. Here we present data that clearly establish that GS₁ in plants is also regulated at the level of transcript turnover and at the translational level. Using a *Glycine max* (soybean) GS₁ transgene, with and without its 3' untranslated region (UTR), driven by the constitutive CaMV 35S promoter in *Medicago sativa* (alfalfa) and *Nicotiana tabacum* (tobacco), we show that the 3' UTR plays a major role in both transcript turnover and translation repression in both the leaves and the nodules. Our data suggest that the 3' UTR mediated turnover of the transcript is regulated by a nitrogen metabolite or carbon/nitrogen ratios. We also show that the 3' UTR of the gene for the soybean GS₁ confers post-transcriptional regulation on a reporter gene. Our dissection of post-transcriptional and translational levels of regulation of GS in plants shows that the situation in plants strongly resembles that in other organisms where GS is regulated at almost all levels. Multistep regulation of GS shows the high priority given by organisms to regulating and ensuring optimal control of nitrogen substrates and preventing overproduction of glutamine and drainage of the glutamate pool.

Keywords

Glutamine synthetase; 3' untranslated region; post-transcriptional regulation; transgenic alfalfa; nitrogen assimilation

Introduction

Glutamine synthetase (GS) is the key enzyme in the assimilation of nitrogen, catalyzing the ATP-dependent condensation of ammonia with glutamate, to yield glutamine. Glutamine synthetase in plants is an octamer and occurs in a number of isoenzyme forms located in either the cytosol (GS₁) or the chloroplast/plastid (GS₂). The GS₁ in leaves and stems is localized in the phloem and functions to generate glutamine for nitrogen transport, while GS₂ is found mainly in the photosynthetic cells. Glutamine synthetase 2 has been proposed

to serve two functions in the leaves: one is the assimilation of ammonia reduced from nitrite in the chloroplasts and the second is the re-assimilation of photorespiratory ammonia (Lam *et al.*, 1995; Taira *et al.*, 2004). Glutamine synthetase 1, on the other hand, functions in assimilating ammonia produced by all other physiological processes.

In plants, where GS₁ is encoded by a small multigene family, it has been well established that the gene members are transcriptionally regulated, with both developmental and environmental cues (Bennett *et al.*, 1989; Gebhardt *et al.*, 1986; Marsolier *et al.*, 1995; Morey *et al.*, 2002; Peterman and Goodman, 1991; Temple *et al.*, 1995; Tingey *et al.*, 1987; Walker and Coruzzi, 1989). However, the absence of correlation between accumulation of transcript and abundance of protein in certain cases suggests that regulation also occurs at a level downstream of transcription (Ortega *et al.*, 1999, 2001; Temple *et al.*, 1996, 1998). One such regulatory step is similar to the two-step turnover mechanism in bacteria and involves the oxidative turnover of the GS protein (Ortega *et al.*, 1999; Suganuma *et al.*, 1999). Based on some of our recent efforts to overexpress GS₁ in *Medicago sativa* (alfalfa), we also postulate that GS in plants is regulated at the level of transcript turnover (Ortega *et al.*, 2001). Alfalfa plants transformed with a *Glycine max* (soybean) or alfalfa GS₁ gene driven by the constitutive cauliflower mosaic virus (CaMV) 35S promoter showed accumulation of the transgene transcript in leaves but not in nodules. The absence of GS₁ transgene transcript in the nodules of these alfalfa transformants suggested that ammonia or an assimilation product was responsible for the instability of the transcript (Ortega *et al.*, 2001). Feeding nitrate to the transformants caused a fivefold drop in the level of transgene transcript and the endogenous GS₁ gene transcript in the leaves. We also showed in this study the complete absence of the GS₁ polypeptide corresponding to the transgene in the leaves of alfalfa transformants grown in nitrogen-deficient conditions, even though the transcripts were made and recruited into polysomes (Ortega *et al.*, 2001).

Glutamine synthetase activity in all organisms studied is precisely controlled and can be rapidly adjusted in response to environmental stimuli, including carbon and nitrogen signals, which are antagonistic (Jiang *et al.*, 1998; Stadtman, 1990). Different regulatory mechanisms control the GS enzyme to ensure optimal utilization of nitrogen substrates (Reitzer and Magasanik, 1987). In bacteria the enzyme is regulated by feedback inhibition by some of the nitrogenous metabolites (Liaw *et al.*, 1993) and by reversible covalent adenylation (Stadtman, 1990), and the structural gene for GS, *glnA*, is subject to transcriptional regulation (Magasanik, 1989). Although normally stable, GS is turned over when cells are starved of nitrogen (Fulks and Stadtman, 1985; Stadtman and Berlett, 1997), indicating that the intracellular level of GS in bacterial cells is also regulated by proteolysis. Glutamine synthetase in *Saccharomyces cerevisiae* (yeast) is also under strong transcriptional regulation responding to glutamine–glutamate shift and purine deprivation (Minehart and Magasanik, 1992). While GS in yeast is not subject to feedback inhibition, it is subject to reversible inactivation as a means to prevent overproduction of glutamine and drainage of the glutamate pool (Mitchell and Magasanik, 1984).

Post-transcriptional mechanisms play a major role in regulation of the expression of some eukaryotic genes (Atwater *et al.*, 1990; Gutiérrez *et al.*, 1999; Hollams *et al.*, 2002). One of the most prominent forms of post-transcriptional regulation in eukaryotic cells is the control of the stability of mRNA (Hollams *et al.*, 2002). Regulation through differential stability of the same mRNA under different metabolic conditions is well documented in eukaryotic cells (Atwater *et al.*, 1990), including in plants (Gutiérrez *et al.*, 1999). Another critical component of post-transcriptional regulation involves modulation of the translation rates of labile mRNAs. These complex regulatory mechanisms have been shown to utilize *cis*-acting elements located in the mRNA sequences. These sequences are frequently, but not exclusively, in the 3' UTR (Hollams *et al.*, 2002).

The focus of this paper is to demonstrate that GS₁ in plants is regulated at a post-transcriptional level and to determine whether this regulatory mechanism is mediated by the nitrogen status. Our experimental approach was to introduce into alfalfa a soybean GS₁ transgene, *GmglNβ₁*, devoid of its transcriptional control elements as a result of engineering it behind the constitutive CaMV 35S promoter, and following its expression under different nitrogen regimens in the leaves and nodules. Furthermore, the GS₁ gene constructs were made to either include (pGSβ₁53) or exclude (pGSβ₁50), its 3' UTR, in order to check if the 3' UTR of the gene has any role in post-transcriptional regulation of the gene. The *GmglNβ₁* gene constructs were also introduced into *Nicotiana tabacum* (tobacco) to determine if the 3' UTR mediated post-transcriptional regulation of GS₁ is limited to legumes. We have also introduced into tobacco the β-glucuronidase (*uidA*) reporter gene, with and without the *GmglNβ₁* 3' UTR at its 3' end, to ascertain if the 3' UTR of the *GmglNβ₁* gene confers post-transcriptional regulation on a reporter gene.

Results

The presence or absence of the 3' UTR in the *GmglNβ₁* gene affects the level of transcript accumulation in the leaves of transgenic alfalfa

To check if the 3' UTR of the *GmglNβ₁* gene plays a role in the post-transcriptional regulation, we have created two *GmglNβ₁* gene constructs, one with and the other without the 3' UTR. These gene constructs were introduced into alfalfa and their expression pattern was followed. The *GmglNβ₁* gene (NCBI accession number AF301590; Morey *et al.*, 2002) containing 1071 bp of coding region and 59 bp of the 5' UTR, with the 3' UTR of 273 bp (GSβ₁53) or 16 bp of 3' UTR (GSβ₁50), was inserted between the CaMV 35S promoter and the nitric oxide synthase (NOS) 3' terminator (pGSβ₁53 and pGSβ₁50, respectively; Figure 1a). These constructs were introduced into alfalfa by *Agrobacterium tumefaciens* mediated transformation. Several independent alfalfa transformants for both pGSβ₁53 and pGSβ₁50 gene constructs were identified by genomic PCR (data not shown) and also analyzed for the transgene transcript (Moguel-Esponda, 2004). In Figure 1b, we show a representative panel of transformants analyzed for different transcripts. Total leaf RNA from control and transformed plants that were nodulated was subjected to Northern blot hybridization using the cDNA of the *GmglNβ₁* gene as a probe to monitor the level of accumulation of transgene transcript. The *GmglNβ₁* probe showed no detectable hybridization signal with the control samples, and hybridized more strongly (about tenfold higher) to a band in the pGSβ₁50 (–3' UTR) plants when compared with the hybridization signal obtained in the pGSβ₁53 (+3' UTR) plants (Figure 1b,c). The blot was also hybridized with a probe for the actin gene (as a representative housekeeping gene) and the chloroplastic/plastidic GS gene (GS₂). The hybridization pattern with the GS₂ and actin probes showed some degree of variation across the different samples, but in general it was fairly uniform and the differences could not be attributed to the presence or absence of the transgene. The approximately tenfold higher level of accumulation of the transgene transcript in the pGSβ₁50 transformants compared with the pGSβ₁53 transformants (Figure 1b,c), suggests that the 3' UTR of the *GmglNβ₁* gene either has a silencing effect on the CaMV 35S promoter or has a destabilizing effect on the transgene transcript.

To ensure that pGSβ₁53 and pGSβ₁50 gene constructs had not undergone sequence rearrangement during the cloning procedures causing differences in the expression pattern between the two classes of transformants, the transgene transcripts isolated from the leaves of pGSβ₁50 and pGSβ₁53 transformants were analyzed. Total RNA isolated from the pGSβ₁50 and pGSβ₁53 transformants was subjected to RT-PCR using primers specific for the CaMV 35S 5' UTR and an anchor primer at the 3' ends of cDNAs. The amplified products were subcloned and sequence analysis of several independent clones confirmed

that the transgene sequences were maintained in the transformants without any changes (data not shown).

The presence or absence of the 3' UTR in *Gmglu* β ₁ transgene constructs has no effect on their transcription rates in the alfalfa transformants

To check if the 3' UTR has a role in the transcriptional control of the CaMV 35S/*Gmglu* β ₁ gene, *in vitro* transcription experiments were performed with nuclei isolated from the leaves of the GS β ₁50 and the GS β ₁53 transformants. The transcripts, synthesized *in vitro* and labeled with ³²P, were hybridized to increasing concentrations of the cloned DNA fragments of genes for the small subunit of ribulose-1,5-bis-phosphate carboxylase (ssRuBCO), the 28S ribosomal RNA, and the *Gmglu* β ₁ transgene immobilized on a nylon membrane. As seen in Figure 2, the *in vitro* transcribed RNA from the two different transformants showed a similar amount of hybridization to the rRNA probe used as control. The *in vitro* labeled transcripts showed a slightly higher hybridization signal to the *Gmglu* β ₁ DNA in the GS β ₁53 transformants compared with the GS β ₁50 transformants, indicating that the presence of the 3' UTR in the CaMV 35S/*Gmglu* β ₁ gene construct does not inhibit its rate of transcription. The higher level of the transgene transcript in the GS β ₁53 transformant could be attributed to a position effect. Hybridization of the *in vitro* transcripts to the ssRuBCO DNA is higher for the GS β ₁50 transformants than the GS β ₁53 transformants. Taken together, the results suggest that the 3' UTR does not affect the rate of transcription of the transgene.

The absence of the 3' UTR in the *Gmglu* β ₁ gene allows for high accumulation of transgene protein in the leaves of alfalfa transformants

To check if the 3' UTR of the *Gmglu* β ₁ gene has any role in the accumulation of the corresponding polypeptide, protein samples from the leaves of several nodulated control plants and independent transformants (including those that were used for RNA analysis in Figure 1) containing either the pGS β ₁53 or pGS β ₁50 gene constructs, were analyzed for levels of GS. The protein samples were subjected to SDS–PAGE followed by immunoblot analysis using GS antibodies (Figure 3a). On SDS–PAGE, the amount of the chloroplastic form (GS₂) appears to be unchanged across all the samples and the cytosolic form (GS₁) is represented as a very minor immunoreactive band in the control plants and pGS β ₁53 transformants, while the pGS β ₁50 transformants showed significantly higher levels of the GS₁ polypeptide.

Since SDS–PAGE did not distinguish between endogenous and transgene GS₁ polypeptide, the leaf protein extracts were also subjected to native gel electrophoresis followed by Western analysis. The major immunoreactive bands, in all the samples on the native gel, represent the GS₂ holoprotein and its faster migrating tetrameric form. Native gel electrophoresis of leaf proteins also showed a unique immunoreactive band in the pGS β ₁50 transformants corresponding to the octamer made up of the transgene polypeptide (GS β ₁, Figure 3). Besides the octamer, the pGS β ₁50 samples also showed an intense fast-migrating band, most likely representing the tetramer made up of the GS β ₁ polypeptide. The high level of the tetrameric form in the pGS β ₁50 transformants would suggest that there is extensive turnover of the holoenzyme that is made up of the GS β ₁ polypeptide. The leaf proteins from the pGS β ₁53 transformants showed a pattern identical to the GS oligomers in the control plants, thus confirming the complete absence of the transgene GS β ₁ polypeptide.

Measurements GS enzyme activity in leaf protein extracts from the two classes of transformants and the control plants also corroborated the data of the native gel analysis of GS. The activity was about 30% higher in the pGS β ₁50 plants compared with control, while the pGS β ₁53 plants had activity similar to control plants (Figure 3b). The measured GS activity is the sum of the chloroplastic GS₂ and the cytosolic GS₁ isoforms, the GS₂ being

the prevalent form in leaves. This increment in total GS activity suggests that the novel GS₁ octamer in the leaves of pGSβ₁50 transformants represents an active enzyme.

Our results demonstrate that the absence of the 3' UTR from the *GmglNβ₁* gene construct allows for the accumulation of the GSβ₁ polypeptide in the leaves of the alfalfa transformants and that these polypeptides assemble into an active holoenzyme. The complete absence of a transgene protein product in the pGSβ₁53 transformants suggests that the 3' UTR plays a role in repression of translation. We can rule out protein turnover as the basis for the absence of the transgene product in the pGSβ₁53 transformants, since the only difference between pGSβ₁50 and pGSβ₁53 is in the presence or absence of the 3' UTR and there is no precedent to suggest that the presence or absence of the 3' UTR influences protein turnover.

The 3' UTR of the *GmglNβ₁* gene plays a major role in the NO₃⁻ mediated turnover of the *GmglNβ₁* transcripts in the leaves of transgenic alfalfa

The data presented thus far suggest that the 3' UTR of the *GmglNβ₁* gene plays a role in determining the steady-state level of the transcript and the protein. To determine if nitrogen metabolites are involved in this 3' UTR mediated regulation, we clonally propagated alfalfa transformants and control plants and treated them with either KNO₃ or KCl to analyze the levels of transgene transcript. The RNA isolated from the leaves of these plants was subjected to quantitative Northern blot analysis using different probes. The rRNA profile was used as an indicator of RNA loads. A representative panel of the Northern blot analysis is shown in Figure 4(a), and the average value of multiple analyses of the *GmglNβ₁* transgene transcript levels for the different transformants is graphically represented in Figure 4(b). A full-length *GmglNβ₁* cDNA probe showed low-level background hybridization to RNA from control leaves treated with KCl and hybridized strongly to bands in the leaf samples from both the pGSβ₁53 and pGSβ₁50 plants treated with KCl. A more specific signal for the *GmglNβ₁* mRNA was observed by using a probe consisting of the last 72 nucleotides of the coding region and the 3' UTR. This probe showed a 2.5-fold higher hybridization signal in the pGSβ₁50 transformants than in the pGSβ₁53 transformants, when grown with KCl. Following nitrate treatment, the hybridization signal with the *GmglNβ₁* probe showed a drop of approximately eightfold in the pGSβ₁53 plants while the pGSβ₁50 transformants showed only a marginal drop in the signal, thus resulting in a transgene transcript level about eight- to tenfold higher in the pGSβ₁50 transformants than in the pGSβ₁53 transformants. The hybridization pattern for the transgene transcript in the two transformants grown with KNO₃ was similar to the pattern seen in nodulated plants (Figure 1) suggesting that the nitrogen status plays a role in the 3' UTR mediated turnover of the GS transgene transcript.

The hybridization profile for the *MsGS100* gene probe was similar for the control plants and the transformants. In all cases a significant drop (about tenfold) in the hybridization signal was seen in all the plants treated with KNO₃. The GS₂ probe, however, showed more than a twofold higher hybridization signal in the plants treated with KNO₃ (control and transgenic) than in the plants fed with KCl, coincidental with the role of GS₂ in the assimilation of ammonia produced by the reduction of nitrate (Zozaya-Hinchliffe *et al.*, 2005). The actin probe, representing a housekeeping gene, showed a more or less uniform hybridization signal across the two treatments for the control and the transformed plants. These results suggest that nitrate or an assimilation product is also responsible for the drop in the level of the endogenous GS₁ (*MsGS100*) transcript, as well as the induction of GS₂ expression in the leaves.

The 3' UTR of the *GmglNβ₁* gene inhibits translation of the *GmglNβ₁* transcript independent of NO_3^- treatment

We next looked at the accumulation pattern for GS proteins in the leaves of the transformants grown under the two nitrogen regimens as described in the previous section, to determine if the profile of accumulation of transgene transcript showed any correspondence to the pattern of accumulation of protein. Protein extracts from the same leaf samples as those used for RNA analysis (Figure 4) were used for SDS-PAGE and native gel electrophoresis followed by Western blot analysis and band quantification. The GS₁ and GS₂ polypeptides are easily distinguishable on SDS-PAGE. However, on native gels the holoenzymes corresponding to the endogenous GS₁ and GS₂ polypeptides migrate very closely, making it difficult to define each isoform. A representative panel of the SDS Western blot analysis is shown in Figure 5(a), while the values of the GS polypeptide levels for the different transformants and control are graphically represented in Figure 5(b). Nitrate treatment showed an increase in the GS₂ polypeptide in all the samples as detected by SDS-PAGE Western analysis, in keeping with the increase in the corresponding transcript level. With regards to the GS₁ polypeptide and holoenzyme, the pGSβ₁53 transformants showed the same amount and profile as the control non-transformed plants (Figure 5) and both showed a drop of about threefold in the accumulation of GS₁ protein with KNO₃ treatment. The pGSβ₁50 transformants, on the other hand, showed much higher accumulation of the GS₁ polypeptide compared with control plants in both the KCl-treated (fourfold) and KNO₃-treated (tenfold) plants. The pGSβ₁50 transformants also showed a novel immunoreactive band on native gels corresponding to an octamer made up of the GmglNβ₁ polypeptide, the band being slightly more broad and diffused in the KCl-treated samples compared with the KNO₃-treated samples. The broad immunoreactive band on native gels in the KCl-treated pGSβ₁50 transformants is probably due to the closely migrating heteromers formed with different proportions of GmglNβ₁ subunits and the endogenous GS₁ polypeptides, while the corresponding band is less heterogeneous in the KNO₃-treated pGSβ₁50 transformants because of a drop in the level of endogenous GS₁ polypeptide. The protein extracts were also used for assays of GS activity (Figure 5c). Measurements of GS activity showed a level of activity approximately twofold higher in KNO₃-treated plants (control and the transformants) than in their KCl-treated counterparts, attributable to the increase in levels of GS₂ activity with KNO₃ treatment. The pGSβ₁50 transformants showed a level of activity approximately twofold higher than the control plants and pGSβ₁53 transformants under both KCl and KNO₃ treatments, and this increase is attributable to the GSβ₁ polypeptide.

Nitrate treatment also caused a drop in the endogenous alfalfa GS₁ polypeptide and holoenzyme in the leaves, as was shown for the endogenous alfalfa GS₁ transcript. This suggests that accumulation of the endogenous GS₁ polypeptide may be a reflection of the level of endogenous GS₁ transcript. The pGSβ₁53 transformant, however, showed no trace of a polypeptide corresponding to the transgene, even though there were significant levels of the transgene transcript, especially under KCl treatment (Figure 4). These results suggest that the 3' UTR mediated repression of translation is independent of the nitrogen status.

The 3' UTR of the *GmglNβ₁* gene has a role in determining the level of *GmglNβ₁* transcript in the nodules of alfalfa. Sequences other than the 3' UTR may also play role in transcript turnover in the nodules

The nodule is the site of N₂ fixation and nitrogen assimilation and exhibits high GS activity. Thus the next logical step was to determine if the *GmglNβ₁* gene was subject to the same 3' UTR mediated post-transcriptional regulation in the nodules as seen in the leaves of the transformants grown under different nitrogen regimens. To simulate the KNO₃/KCl treatment in the leaves with that of nodules, plants were clonally propagated in Magenta boxes and inoculated with the wild type (WT) and nitrogen fixation deficient (Fix⁻) strains

of *Sinorhizobium meliloti*. While the nodules infected with the WT strain would have a high concentration of ammonia or/and its assimilation product, the nodules infected with the nitrogen-fixation mutant would be nitrogen deficient. Thus any difference in the pattern of turnover of the *Gmglb₁* transcript between the two nodule types would be indicative of the role of the nitrogen status in this regulatory step.

Leaves and nodules from control and transgenic alfalfa plants with the pGS β ₁50 or pGS β ₁53 gene constructs were harvested 5 weeks after inoculation with the WT or Fix⁻ strains of *S. meliloti* and used for RNA extraction. The RNA was subjected to Northern blot analysis and hybridized with a full-length cDNA for the *Gmglb₁* gene as a probe. Results showed significant differences in accumulation of GS₁ transgene transcript in the nodules between the plants inoculated with the WT and Fix⁻ strains of *S. meliloti* (Figure 6). The probe showed a low level of hybridization to a composite of closely migrating multiple bands in the nodules from control plants, suggesting some level of cross-hybridization to the endogenous GS₁ mRNA. The WT nodules showed no transcript for the transgene in the pGS β ₁53 transformants. Since the CaMV 35S promoter is functional in the nodules (Bagga *et al.*, 1992; Ortega *et al.*, 2001), the absence of the transgene transcript in the WT nodules of the pGS β ₁53 transformants is suggestive of post-transcriptional regulation. In contrast to the WT nodules, the Fix⁻ nodules on the pGS β ₁53 transformants showed a significant accumulation of the transgene transcript at a level two- to threefold higher than in the leaves (Figure 6), thus reinforcing our earlier finding that the nitrogen status has a role in the turnover of the *Gmglb₁* gene transcript. The WT nodules on the pGS β ₁50 transformants showed low-level accumulation of the transgene transcript, while an increase in the transcript level of more than tenfold was seen in the pGS β ₁50 Fix⁻ nodules. The data on transcript accumulation presented here suggest that the presence of the 3' UTR in the *Gmglb₁* transgene does not allow for the accumulation of the corresponding transcript in the WT nodules. However, the huge increase in the accumulation of the transgene transcript in the Fix⁻ nodules compared with the WT nodules for both the transformants suggests that sequences other than the 3' UTR in the *Gmglb₁* gene also play a role in the turnover of the transcript, this step being regulated by the availability of fixed nitrogen.

The RNA blot was also probed with the nodule-enhanced endogenous GS₁ (*MsGS13*) gene-specific probe. The pGS13 probe (Temple *et al.*, 1995) showed no difference in the level of pGS13 transcript between the WT and Fix⁻ nodules in control and transformed plants, suggesting that the expression of the pGS13 gene is not regulated, either transcriptionally or post-transcriptionally, by the nitrogen status of the nodules.

The 3' UTR prevents the *Gmglb₁* transcript from being translated in transgenic alfalfa nodules

The leaves of pGS β ₁53 transformants showed accumulation of the transgene transcript but no trace of the corresponding transgene polypeptide (Figures 1 and 3). The leaves of pGS β ₁50 transformants, however, showed accumulation of a novel GS₁ holoenzyme (Figure 3). To check if the same phenomenon holds true in the nodules, nodule proteins were analyzed for GS₁ polypeptide and holoenzyme. The protein samples from the nodules of control and transformed plants inoculated with the WT and Fix⁻ strains were subjected to SDS-PAGE followed by Western blotting using the GS₁ antibodies (Figure 7a), and used to measure GS activity (Figure 7b). The level of GS₁ polypeptide, and GS activity in general, was higher in the WT nodules than in the Fix⁻ nodules and no difference was seen in the GS₁ polypeptide level in the WT nodules between the transformants and the control plants. In contrast, the Fix⁻ nodules of pGS β ₁50 transformants showed a higher level of GS₁ polypeptide and GS activity than the Fix⁻ nodules of pGS β ₁53 transformants and control plants.

To differentiate between the endogenous GS₁ polypeptide and the transgene GS₁ polypeptide, the protein samples from the nodules were also subjected to native gel electrophoresis followed by Western blotting using the GS₁ antibodies. As seen in Figure 7(c), the pGSβ₁50 transformants showed the presence of a novel banding pattern in both the WT and Fix⁻ nodules consistent with the presence of novel GS₁ subunits in addition to the endogenous GS₁ subunits assembled into heteromeric holoprotein.

It is important to point out that while both the WT and Fix⁻ nodules on the pGSβ₁50 plants showed the presence of the novel GS holoproteins, the nodules on the pGSβ₁53 transformants showed no trace of these holoproteins even though the amount of transgene transcript in the pGSβ₁53 Fix⁻ nodules was about five times higher than in the pGSβ₁50 WT nodules (Figure 6). These results suggest that the 3' UTR plays a major role in the repression of translation of the *GmglNβ₁* transcript in the nodules and that this event is independent of nitrogen fixation, similar to the NO₃⁻ independent translation repression of *GmglNβ₁* transcript in the leaves of pGSβ₁53 transformants (Figure 5).

The 3' UTR of the *GmglNβ₁* gene affects transcript stability and the translation of the *GmglNβ₁* transcript in transgenic tobacco

To check whether the effect of the 3' UTR of *GmglNβ₁* on transcript turnover and translation repression is unique to alfalfa, we compared the level of transcript and polypeptide corresponding to the transgene in tobacco plants transformed with the two gene constructs. Independent transformants of tobacco with the two different gene constructs and control plants growing under nitrogen-sufficient conditions were used for RNA extraction. The RNA was subjected to Northern blot hybridization using the full-length cDNA clone for the *GmglNβ₁* gene as a probe, and a representative panel is shown in Figure 8(a). The *GmglNβ₁* probe did not show any hybridization to the RNA from the control plants but showed hybridization to RNA from both sets of transformants. The hybridization signal in the pGSβ₁50 transformants was consistently higher than in the pGSβ₁53 transformants by three- to fivefold, mimicking the pattern seen in the alfalfa transformants (Figure 1). The difference in the level of transgene transcript between the two classes of tobacco transformants, however, is smaller than between the two classes of alfalfa transformants.

Protein extracts from the leaf tissue of three of each of the same transformants used for RNA analysis and control plants were subjected to both SDS-PAGE and native gel electrophoresis followed by Western analysis using GS₁ antibodies. As seen in Figure 8(b), the pGSβ₁50 transformants showed a significantly higher accumulation of the GS₁ polypeptide compared with both control and the pGSβ₁53 transformants, and showed a novel GS₁ holoprotein on native gels. The pGSβ₁53 transformants showed a very slight increase in the level of GS₁ polypeptide compared with control plants and showed a GS₁ octamer with a migration pattern different from that seen in controls or the pGSβ₁50 transformants. The difference in migration among the GS₁ octamers in the control plants and the two classes of transformants is a reflection of differences in the composition of the octamer. The results indicate that while the GS₁ holoprotein in the pGSβ₁50 transformants is mostly made up of the *GmglNβ₁* subunits, the GS₁ holoprotein in the pGSβ₁53 transformants is mostly made up of the endogenous GS₁ subunits with a very small fraction of *GmglNβ₁* polypeptide.

Native gel Western analysis also showed the presence of GS tetramers with differences in the migration pattern between the pGSβ₁50 and pGSβ₁53 transformants and the control non-transformed samples, confirming the differences in the subunit composition. Measurements of GS activity (Figure 8c) showed a good correlation between GS activity and the level of GS holoenzyme. While the pGSβ₁53 transformants showed a slightly increased level of GS activity over controls, the pGSβ₁50 transformants showed an increase of almost 1.5-fold.

Taken together, the results suggest that the pGS β_1 53 gene construct is transcribed in transgenic tobacco and the transcript is translated, but at a highly reduced level compared with the pGS β_1 50 transcript. We conclude that, as in the case of alfalfa, the 3' UTR of the *Gmglu* β_1 gene shows the same effect on transcript turnover and translation repression in transgenic tobacco, thus emphasizing the universality of the post-transcriptional regulation of GS₁ in plants.

The 3' UTR of the *Gmglu* β_1 gene affects transcript stability of the β -glucuronidase reporter gene transcript in transgenic tobacco

To confirm that the 3' UTR of the *Gmglu* β_1 gene is an important component for regulation of transcript stability, a reporter gene construct was made by cloning the *Gmglu* β_1 3' UTR downstream of the bacterial β -glucuronidase gene (*uidA*) under the control of the CaMV 35S promoter (Figure 9). The *uidA* gene constructs with and without the *Gmglu* β_1 3' UTR (*uidA::glu* β_1 3' UTR and *uidA*, respectively) were introduced into tobacco. Several independent transformants for each gene construct were then analyzed for the GUS transcript by Northern blot hybridization. As seen in Figure 9, while the transformants with *uidA* gene construct showed strong hybridization to a band of about 2 kb with the *uidA* gene probe, the transformants with the *uidA::glu* β_1 3' UTR showed a lower level of hybridization to multiple bands. The multiple bands are attributable to the utilization of multiple polyadenylation signals in the *Gmglu* β_1 3' UTR and the NOS terminator (as indicated in Figure 9a). All the hybridizing bands were quantified and it was found that the total of all hybridizing bands in the *uidA::glu* β_1 3' UTR transformants is about three times lower than in the *uidA* transformants, suggesting that the *Gmglu* β_1 3' UTR has the same effect on the GUS transcript stability as it has on the *Gmglu* β_1 coding sequence.

Discussion

In this paper we have shown that a GS₁ gene (*Gmglu* β_1) used as a transgene is regulated post-transcriptionally at the levels of both transcript stability and protein accumulation. We have ruled out transcriptional regulation as the basis for the difference in the steady-state level of the transcript in the two classes of transformants because nuclear run-on experiments clearly established that the rate of synthesis of transgene transcript was essentially the same in both (Figure 2). We have further demonstrated that the post-transcriptional regulation is mediated by the 3' UTR of the gene (Figure 9). Both the tobacco and alfalfa transformants with the *Gmglu* β_1 gene construct without its 3' UTR (pGS β_1 50) showed significantly higher levels of the transgene transcript and GS₁ polypeptide accumulation compared with the transformants with the *Gmglu* β_1 gene construct with its 3' UTR (pGS β_1 53, Figures 1 and 3).

Our data also suggest that the 3' UTR mediated turnover of the *Gmglu* β_1 transcript is regulated by the nitrogen status or, as in the case of bacteria, by the C/N ratio. The fact that the endogenous alfalfa (*MsGS100*; Temple *et al.*, 1995) transcript also exhibits reduced accumulation mediated by nitrate (Figure 4) would further suggest that post-transcriptional regulation may not be limited to just the *Gmglu* β_1 gene but may extend to other GS₁ genes. The fact that *Gmglu* β_1 is regulated similarly in two different plant systems, alfalfa and tobacco, suggests that the post-transcriptional regulation of GS₁ is probably of universal significance in plants.

In bacteria, different regulatory mechanisms control the GS enzyme to ensure optimal utilization of nitrogen substrates (Reitzer and Magasanik, 1987). Thus it is not surprising that GS in plants is also regulated at multiple levels. Because ammonia is toxic, it has to be assimilated as soon as it is produced. Thus, it is a strategy for plants to have GS either already made or ready to be made as the need arises. Moreover, ammonia assimilation is

dependent on the availability of empty carbon skeletons. If carbon skeletons are limiting or the C/N ratio is not adequate, it becomes critical for the plant to terminate the synthesis of GS at an early stage and thus reduce the energy and carbon expenditure required for ammonia assimilation. Probably the ideal conditions for synthesizing GS are when adequate carbon skeletons are available to load up with the nitrogen.

Treatment of pGS β_1 53 transformants with NO $_3^-$ resulted in a fivefold drop in the level of the transgene transcript; this is not related to any effect of NO $_3^-$ on the CaMV 35S promoter (Ortega *et al.*, 2001). This suggests that the drop in the steady-state level of the pGS β_1 53 transcript in the transformants is probably an attribute of RNA turnover and not its synthesis. Moreover, the fact that the pGS β_1 50 transformants do not exhibit a NO $_3^-$ mediated drop in the level of transgene transcript (Figure 4) suggests that the 3' UTR of the *Gmgl β_1* gene is the regulator of transcript turnover. Based on these results, we could also infer that the 3' UTR mediated turnover of the transcript is regulated by NO $_3^-$ or its assimilation product. This is further supported by the fact that no transcript for the pGS β_1 53 transgene is seen in the WT nodules while there is significant accumulation of the transcript in the Fix $^-$ nodules (Figure 6).

It is interesting to point out that nitrate treatment of alfalfa plants also led to a dramatic drop in the level of transcript corresponding to the endogenous gene *MsGS100* in the leaves (Figure 4). Although we cannot rule out the possibility that this nitrate mediated drop in the level of the *MsGS100* transcript is an attribute of promoter function, we speculate that, like the *Gmgl β_1* gene, the *MsGS100* gene is also regulated at the level of transcript turnover. However, it is important to point out that the nodule-enhanced alfalfa GS $_1$ gene, *MsGS13* (Temple *et al.*, 1995), is not regulated like the *Gmgl β_1* gene in the WT and Fix $^-$ nodules, suggesting that there may be GS $_1$ gene member-specific regulation at a post-transcriptional level. Sequence alignment of the 3' UTRs of the *MsGS100* gene from alfalfa and the soybean *Gmgl β_1* has revealed regions that are conserved in the 3' UTR of the two GS $_1$ genes (data not shown). The 3' UTRs of the two GS $_1$ genes also showed extensive secondary structure. Moreover, we have also identified a region in the 3' UTR of the GS $_1$ genes with high sequence similarity to the region III in the rice α -amylase 3' UTR which has been implicated to have a role in turnover of mRNA (Chan and Yu, 1998). Other GS $_1$ gene sequences, including the *Gmgl β_2* (Morey *et al.*, 2002), and the *Pvgl β* (Gebhardt *et al.*, 1986) genes, also show similarity to the *Gmgl β_1* gene at the 3' UTR. Experiments are in progress to test whether the 3' UTR of the *MsGS100* gene plays a role in the regulation of the gene.

Whether the nitrogen metabolite functions directly or via a *trans*-factor in regulating the 3' UTR mediated turnover of the transcript is not known at this time. The 3' UTRs of many RNAs are known to bind to regulatory proteins and these RNA–protein complexes are known to control the stability of the RNA (Buzby *et al.*, 1999; Casey *et al.*, 1988; Lai *et al.*, 1999; Loflin *et al.*, 1999; Müllner and Kühn, 1988). However, there is also evidence in the literature of metabolite-binding RNA structures called riboswitches that serve as genetic control elements for certain mRNAs (Winkler *et al.*, 2002). While genetic control by metabolite-binding mRNAs is widespread in prokaryotes, some recent data suggest that riboswitches are also present in the genes of eukaryotes (Sudarsan *et al.*, 2003). It has been proposed that when present in the 3' UTR a riboswitch may control mRNA processing or stability.

Our data further suggest that besides playing a role in transcript turnover, the 3' UTR of the *Gmgl β_1* gene may also play a role as a translational repressor. The pGS β_1 53 alfalfa transformants, while showing accumulation of the transgene transcript (Figures 4 and 6), did not show accumulation of any GS $_1$ polypeptide or holoenzyme corresponding to the

transgene (Figures 5 and 7). Furthermore, we had previously shown that the *Gmglu* β ₁ transcript was recruited into polysomes but there was no accumulation of the transgene polypeptide in the alfalfa transformants (Ortega *et al.*, 2001), thus suggesting translation repression at stages beyond polysome loading. We rule out protein turnover as the basis of the difference in the steady-state levels of the polypeptide between the pGS β ₁53 and pGS β ₁50 transformants because the two constructs differ only in the presence or absence of the 3' UTR and there is no precedent to suggest that the presence or absence of the 3' UTR could influence protein turnover.

Alfalfa pGS β ₁53 transformants grown under different nitrogen conditions showed no accumulation of the transgene polypeptide (Figures 5 and 7). This suggests that the 3' UTR mediated repression of translation is independent of the nitrogen status. The nitrogen-independent regulation of the 3' UTR mediated translation repression is further supported by the fact that even though there is significant accumulation of the GS β ₁53 transgene transcript in the Fix⁻ nodules, no polypeptide or holoenzyme corresponding to the GS β ₁53 transgene could be detected in these Fix⁻ nodules (Figures 6 and 7). On the other hand, the WT nodules of pGS β ₁50 transformants, while showing levels of transgene transcript many times lower than the Fix⁻ nodules of pGS β ₁53 transformants, showed significant accumulation of the transgene polypeptide (Figures 6 and 7). It thus appears that the 3' UTR mediated translation repression of the *Gmglu* β ₁ transcripts, unlike the regulatory step involving transcript turnover, is not regulated by the nitrogen status. These results also suggest that 3' UTR mediated transcript turnover and translation of *Gmglu* β ₁ are probably independent of each other even though translation has been shown to play an important role in controlling degradation of mRNA (Hentze and Kulozik, 1999; Maquat, 2004). A tenfold higher level of GS β ₁50 transgene transcript in the Fix⁻ nodules compared with the WT nodules for the pGS β ₁50 transformants suggests that sequences other than the 3' UTR respond to the nitrogen status in the nodules. It is possible that sequences in either the 5' UTR or in the coding region also play a role in the post-transcriptional regulation of this gene. The regulatory sequence involved in the post-transcriptional regulation of the cystathionine- β -synthase gene in Arabidopsis has been shown to be the amino acid sequence encoded by the first exon (Chiba *et al.*, 1999). In the ferredoxin-1 transcript, the *cis*-acting determinant controlling mRNA stability and translation is located in the 5' UTR and in the 5' coding region (Petracek *et al.*, 1998).

The tobacco pGS β ₁53 transformants showed low levels of GS₁ holoenzyme with a different mobility from that seen in control plants (Figure 8). This implies that the holoprotein contains a mixture of GS₁ transgene product and endogenous GS₁ subunits. However, the level of the transgene polypeptide is many times lower in the tobacco pGS β ₁53 transformants than in the pGS β ₁50 transformants. This indicates that the 3' UTR mediated transcript degradation and translation repression of *Gmglu* β ₁ is not restricted to alfalfa but also occurs in other plant systems. It is important to point out that both the transgene transcripts (GS β ₁53 and GS β ₁50) amplified from the alfalfa transformants by RT-PCR were transcribed and translated in *Escherichia coli* and showed no difference in the level of accumulation of the transgene GS₁ polypeptide (data not shown). Moreover, GS β ₁53 transcripts isolated by hybrid selection from transformed alfalfa plants were translatable in the *in vitro* rabbit reticulocyte system (Ortega *et al.*, 2001). Based on these results we can infer that the 3' UTR mediated turnover of the transcript and translation repression require some plant factor.

Overexpression of GS₁ in certain plants has been shown to be accompanied by increased growth and plant performance (Fuentes *et al.*, 2001; Ortega *et al.*, 2004). However, just introducing a GS₁ transgene driven by the appropriate promoter is not always sufficient for overexpressing GS₁ (Ortega *et al.*, 2001). The expression of GS is highly regulated and

occurs in many steps (Figure 10), thus making it crucial that we understand the regulation of GS before manipulating the gene for expression in a transgenic system. The findings in this paper are very significant since the ability of the 3' UTR to decrease levels of transcript of a reporter gene indicates that it has a role in the post-transcriptional regulation of a GS₁ gene.

Experimental procedures

Gene constructs

Standard procedures were used for all recombinant DNA manipulations (Sambrook *et al.*, 2001). Cloning and isolation of the *G. max* (soybean) *Gmgluβ₁* cDNA clone (NCBI accession number AF301590) has been previously reported (Ortega *et al.*, 2001). This cDNA corresponds to an ammonia-inducible GS₁ isoform of soybean (Miao *et al.*, 1991; Ortega *et al.*, 1999). The *Gmgluβ₁* cDNA contains a 272 bp 3' UTR. The p*Gmgluβ₁* clone was digested with *Bsp*MI and *Pst*I to remove a 254 bp fragment containing most of the 3' UTR to create the plasmid p*Gmgluβ₁*50. The 1145 bp *Gmgluβ₁* cDNA, without the 3' UTR, was released as a *Kpn*I and *Cl*aI fragment and subcloned into pMON 316 in between the CaMV 35S promoter and the NOS 3' terminator to produce the gene construct pGSβ₁50. The construction of the plasmid pGSβ₁53 with the *Gmgluβ₁* cDNA, including its 3' UTR, in between the CaMV 35S promoter and the NOS 3' terminator has been previously described (Ortega *et al.*, 2001).

A gene fusion of the bacterial β-glucuronidase reporter gene (*uidA*) and the 3' UTR of the *Gmgluβ₁* gene was made by engineering the binary plasmid pBI 121 (Clontech BD Biosciences, San Jose, CA, USA; NCBI accession number AF485783) which contains a CaMV35/*uidA*/NOS 3' gene construct. The 272 bp 3' UTR of *Gmgluβ₁* was amplified by PCR from plasmid DNA with primers flanking the region that also include *Sst*I sites. The 3' UTR fragment was cloned and sequenced. The fragment was then isolated and inserted into the *Sst*I site of pBI 121 plasmid downstream the *uidA* reporter gene.

Plant transformation

The pGSβ₁53 and pGSβ₁50 plasmids were mobilized from *E. coli* into *A. tumefaciens* strain A206 containing the Ti plasmid pTi-T37Ase by triparental mating as described (Rogers *et al.*, 1987). The binary vector pBI 121 (CaMV35/*uidA*/NOS 3') and its derivative plasmid with the CaMV35/*uidA*/*gluβ₁* 3' UTR/NOS 3' gene construct were transformed into *A. tumefaciens* strain A4405. Transformation of *M. sativa* (alfalfa) Regen-SY was carried out as previously described (Ortega *et al.*, 2001). *Nicotiana tabacum* (tobacco) cv. Xanthi plants were transformed using the leaf disk transformation procedure (Horsch *et al.*, 1985). The GS₁ transformants were identified by PCR using primers specific for the *Gmgluβ₁* 5' UTR (5'-AAG GGT CTT TGC TTG ATT TTG G-3') and the NOS 3' UTR (5'-CTA GTA ACA TAG ATG ACA CCG C-3'). All the transformants and control plants have an identical background, except for the presence and position of the transgenes in the genome, since all transformation was performed on the same clonal material.

Plant material

Transformed plants were transferred to pots with soil and maintained in the greenhouse. With the exception of nitrogen-controlled experiments, all alfalfa plants were nodulated with aWT *S. meliloti*. For experiments with nitrogen-controlled conditions, plants were vegetatively propagated from the original transformants. Plant cuttings were planted on sterile vermiculite in Magenta boxes as described previously (Sengupta-Gopalan and Pitas, 1986) and maintained with sterile 0.25× Hoagland's nutrient solution containing 1 mM NH₄NO₃ until they developed a root system (about 4 weeks after planting). To obtain alfalfa nodules, the transformed and control rooted cuttings were inoculated with either WT *S.*

meliloti strain 1021 or *nifH::Tn5* derived strain 1491 deficient in nitrogen fixation (Hirsch *et al.*, 1983). Plants were maintained under sterile conditions with 0.25× Hoagland's nutrient solution supplemented with 0.125 mM NH_4NO_3 . Nodules were harvested 35 days after inoculation.

For nitrogen feeding experiments, vegetatively propagated plants in Magenta boxes were grown with sterile 0.25× Hoagland's nutrient solution containing 1 mM NH_4NO_3 for 2 weeks after they had developed a root system. At this stage the plants were provided with sterile 0.25× Hoagland's nutrient solution containing either 10 mM KNO_3 or 10 mM KCl for 4 days. Tissues were harvested in liquid nitrogen and used for RNA and protein analysis. The nitrate treated and non-nitrate treated plants were clonal material. All experiments described in this paper were performed on different transformants and were done at least three times. Only the results from representative sets of experiments are shown here.

RNA analysis

Total RNA was isolated from leaves and nodules by the LiCl precipitation procedure (De Vries *et al.*, 1982). Ribonucleic acid samples were fractionated in 1.35% agarose/formaldehyde gels and transferred to Zeta-Probe membranes according to the manufacturer's instructions (Bio-Rad, Hercules, CA, USA). Blots were hybridized to ^{32}P -labeled probes prepared from plasmid inserts labeled by the random primer method as described (Temple *et al.*, 1998). To monitor the expression of the *GmglN* β_1 gene, the 1.4 kb full-length cDNA of the *GmglN* β_1 gene was used as a probe. A more specific probe for the *GmglN* β_1 gene that corresponds to a *HaeIII/EcoRI* fragment (Figure 1) and contains the last 72 nucleotides of the coding region and the 3' UTR was also used. For the analysis of the *GS* $_1$ alfalfa genes, 3' UTR probes specific for the constitutive *GS* $_1$ gene (*MsGS100*) and the nodule-enhanced *GS* $_1$ gene (*MsGS13*) were used. The expression of the chloroplastic *GS* $_2$ isoform was monitored by using a full-length alfalfa cDNA clone for *GS* $_2$ (Zozaya-Hinchliffe *et al.*, 2005). A cDNA clone for actin from soybean (*G. max* SAC3) was used as a heterologous probe to monitor the actin transcript level. Unless otherwise indicated, rRNA was stained with SYBR Gold (Molecular Probes, Eugene, OR, USA) to check for RNA loads. To monitor the expression of the β -glucuronidase gene, a 1.9 kb *BamHI/SstI* fragment corresponding to the full-length cDNA of the *uidA* gene (Figure 9) was used as a probe. Membranes were pre-hybridized for a minimum of 4 h and hybridized for 20–24 h in 50% formamide (v/v), 5× SSC, 5× Denhardt's solution, 50 mM sodium phosphate (pH 7.0), 0.5% SDS (w/v) and 0.1 mg ml $^{-1}$ denatured salmon sperm DNA at 42°C. Following hybridization the filters were washed three times with 2× SSC, 0.5% SDS (w/v) at 54°C, followed by two washes with 0.5× SSC, 0.5% (w/v) SDS at 54°C for 20 min each, and exposed to X-ray film. The hybridization signals were subjected to band quantification analysis using KODAK1D analysis software (Kodak Scientific Imaging Systems, Rochester, NY, USA).

Rates of transcription of the *GmglN* β_1 gene in the transgenic plants were determined by a nuclear run-off transcription assay performed as described (Greenberg and Bender, 1997). Nuclei were isolated from 10 g of fresh leaf tissue following separation on Percoll gradients as reported (Luthe and Quatrano, 1980). Newly transcribed ^{32}P -labeled RNA was hybridized to a 850 bp *BamHI* fragment from the p*GmglN* β_1 50 plasmid which carries the soybean *GmglN* β_1 gene without the 3' UTR, and to fragments corresponding to the gene encoding the small subunit of the alfalfa ribulose-1,5-bisphosphate carboxylase and the ribosomal RNA gene from alfalfa. Hybridization and washing conditions were the same as for Northern blot analysis, except that the temperature for hybridization was 50°C.

Protein extraction and GS enzyme activity assay

All procedures were carried out at 4°C. Plant tissues were ground in liquid nitrogen and homogenized with five volumes of cold extraction buffer: 50 mM Tris-HCl pH 8.0, 20% glycerol (v/v), 5% ethylene glycol (v/v), 1 mM MgCl₂, 1 mM DTT, 1 mM EDTA and a protease inhibitors cocktail (Roche Applied Science, Indianapolis, IN, USA). The homogenate was centrifuged for 15 min at 20 000g and desalted in Sephadex G25 columns against desalting buffer [62.5 mM Tris-Cl pH 6.8, 20% glycerol (v/v), 5% ethylene glycol (v/v), 1 mM DTT, 1 mM EDTA and protease inhibitors]. Protein concentration was measured by the Bradford protein assay (Bio-Rad) using bovine serum albumin as the protein standard. The activity of GS was measured spectrophotometrically at 500 nm by the transferase assay (Ferguson and Sims, 1971). Transferase units were calculated from a standard curve of γ -glutamyl hydroxamate. Activity is reported as μmol of γ -glutamyl hydroxamate produced $\text{min}^{-1} \text{mg}^{-1}$ protein at 30°C. The GS activity data are averages of at least three independent experiments.

Polyacrylamide gel electrophoresis

Two different PAGE systems were utilized: SDS-PAGE using 12% acrylamide (w/v) gels and a non-denaturing (native) PAGE performed in 7.5% acrylamide (w/v) slab gels, in which the pH of the resolving gel was 8.15. The amount of protein loaded per lane was 5 μg for denaturing gels and 10 μg for the non-denatured protein samples. Denatured proteins were fractionated at 100 V for 16 h. For the native gels the proteins were fractionated at 125 V for 16 h at 4°C.

Immunodetection of GS

For immunoblot analysis, proteins were electroblotted onto nitrocellulose. Glutamine synthetase was detected using an anti-GS antibody raised in rabbits against a recombinant soybean GS β_1 protein expressed in *E. coli* using a glutathione-S-transferase expression system (Amersham Biosciences, Piscataway, NJ, USA). Cross-reacting polypeptides were made visible using an alkaline phosphatase-linked second antibody employing nitroblue tetrazolium and 5-bromo-4-chloro-3-indoyl-phosphate. Glutamine synthetase immunoreactive bands were quantified with the KODAK 1D image analysis software (Kodak Scientific Imaging Systems). Experiments were repeated at least three times. Only representative experiments are presented.

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Figure 1. Transcript analysis in alfalfa transformed with the *GmglNβ*₁ gene with or without its 3' UTR

(a) The *GmglNβ*₁ cDNA with and without its 3' UTR was engineered between the CaMV 35S promoter and the NOS 3' terminator to make the gene construct pGSβ₁53 and pGSβ₁50, respectively.

(b) Total RNA (20 μg) from leaves of control plants and independent transformants for the GSβ₁53 and the GSβ₁50 gene constructs grown under nitrogen-sufficient conditions were subjected to Northern blot hybridization using the *GmglNβ*₁ full-length cDNA as a probe. Sample numbers correspond to the control and transgenic lines from which plants were randomly selected. The Northern blot was also hybridized to ³²P-labeled probes corresponding to actin and the chloroplastic GS isoform (MsGS₂) as controls for loadings. The 28S rRNA in the SYBR Gold stained gel is also shown. (Only a subset of the samples analyzed that were randomly selected is shown here.)

(c) The hybridization bands corresponding to the *Gmgl β ₁*, GS₂ and actin mRNAs from samples from at least three different independent experiments were quantified and plotted. Bars represent the average $\pm$ SE.

Figure 2. Hybridization of 32 P-labeled *in vitro* synthesized nuclear transcripts from the GS β ₁₅₃ and GS β ₁₅₀ transformants to DNA probes

Transcription run-off reactions were made with nuclei isolated from transgenic plants transformed with the *Gmglu β ₁* gene, with (GS β ₁₅₃) and without (GS β ₁₅₀) its 3' UTR.

(a) Labeled transcripts from the two transformants were hybridized to increasing concentrations of DNA fragments for the gene encoding the small subunit of the ribulose 1,5-bisphosphate carboxylase (RuBCO), the ribosomal RNA gene and the soybean *glu β ₁* transgene (*Gmglu β ₁*).

(b) The hybridization signals were quantified and the band intensities were plotted.

Figure 3. Protein analysis of alfalfa transformed with the *Gmgluβ₁* gene with and without its 3' UTR

(a) Five (denatured protein) or 10 (non-denatured protein) μg of protein from leaf extracts of alfalfa control plants and independent transformants for the *Gmgluβ₁* gene with (GSβ₁53) and without (GSβ₁50) the 3' UTR were subjected to either SDS-PAGE or native gel electrophoresis, followed by immunostaining with the GS antibody. Sample numbers correspond to the control and transgenic lines from which plants were randomly selected. The bands for chloroplastic (GS₂) and cytosolic (GS₁) forms are indicated. The octameric (8mer) and tetrameric (4mer) forms of GS were identified by their migration rate (M_r), according to standards included in the gel (not shown). Only a subset of the samples analyzed are shown here.

(b) GS activity was measured in the leaf protein samples from control and the transformed plants and the average values ± SD are presented.

Figure 4. Effect of nitrate feeding on the accumulation of the GS₁ transcripts in alfalfa plants transformed with the *Gmgl β ₁* gene

(a) Total RNA was isolated from leaves of clonally propagated control plants and plants transformed with the *Gmgl β ₁* gene with (GS β ₁53) or without (GS β ₁50) its 3' UTR that were fed either 10 mM KCl or 10 mM KNO₃. The RNA (20 μ g per lane) was then fractionated by gel electrophoresis, blotted and hybridized to ³²P-labeled probes: the full-length cDNA of the *Gmgl β ₁*, the 3' UTR of the *Gmgl β ₁*, the 3' UTR of the endogenous alfalfa GS₁ isoform (*MsGS100*) and a full-length cDNA for actin. SYBR Gold stained ribosomal RNA is shown as a loading standard. Sample numbers correspond to the control and transgenic lines from which plants were clonally propagated.

(b) The experiment was performed three times with different transformants and the average of the *Gmgl*β₁ band intensity ± SD from multiple measurements is represented as bars.

Figure 5. Protein analysis in alfalfa plants transformed with the soybean *Gmglb₁* gene Alfalfa control plants and plants transformed with the soybean *Gmglb₁* gene, either with (GS β_1 53) or without (GS β_1 50), were clonally propagated, while one set was treated with 10 mM KCl, a second set was treated with KNO₃.

(a) Total protein (5 μg) from the leaves of these plants was subjected to SDS-PAGE followed by Western blot analysis using an antibody against the soybean GS β_1 protein. GS₁ and GS₂ are the cytosolic and chloroplastic forms of GS, respectively. Ten micrograms of leaf protein from the same samples was subjected to non-denaturing PAGE (Native) followed by Western blot analysis using anti-GS antibodies. The octameric forms of GS are indicated. Sample numbers correspond to the control and transgenic lines from which plants were clonally propagated.

(b) The GS band intensity is represented as bars.

(c) Activity of GS transferase was measured in the leaf extracts. The values of GS activity are the average $\pm$ SD from at least three different experiments.

Figure 6. Analysis of GS₁ transcripts in control and GS₁ transgenic alfalfa plants inoculated with either a WT *Sinorhizobium meliloti* or a mutant deficient in nitrogen fixation
 Total RNA was isolated from the leaves and nodules of clonally propagated control (non-transformed) and transgenic alfalfa plants transformed with the soybean *GmglN*β₁ gene with (GSβ₁53) or without (GSβ₁50) its 3' UTR, that were inoculated with either a *S. meliloti* *nifH*⁻ mutant (Fix⁻) or a WT strain. Ribonucleic acid (20 μg) was hybridized in Northern blots to ³²P-labeled probes for the soybean *GmglN*β₁ cDNA and the 3' UTR of the nodule enhanced GS₁ gene (*MsGS13*). Numbers correspond to the control and transgenic lines from which plants were clonally propagated. An arrow indicates the transcript for the GSβ₁50 gene construct. The SYBR Gold stained gel is shown as standard for RNA loadings. A panel representative of three experiments is presented here.

Figure 7. Western analysis of the GS protein in alfalfa from clonally propagated control and GS₁ transgenic plants inoculated with either a WT *S. meliloti* or a mutant deficient in nitrogen fixation. Leaf and nodule protein were extracted from the same alfalfa non-transformed (control) and transgenic plants transformed with either the pGSβ₁53 or the pGSβ₁50 gene constructs that were inoculated with either a *S. meliloti* *nifH*⁻ mutant (Fix⁻) or a WT strain, as in Figure 5. The leaf and nodule proteins were analyzed for GS activity and GS protein

(a) Five micrograms of leaf and nodule protein samples was fractionated by SDS-PAGE and subjected to immunoblot analysis using an antibody raised against the soybean GSβ₁. The cytosolic (GS₁) and chloroplastic (GS₂) forms of GS are indicated.

(b) Protein samples were assayed for GS activity by the transferase reaction; the values presented are the average ±SD from three independent experiments.

(c) Ten micrograms of protein from the nodule samples shown in panel A was also subjected to native gel electrophoresis followed by immunodetection with the anti-GS antibody.

Figure 8. Ribonucleic acid and protein analysis in tobacco transformed with the *Gmglnβ₁* gene
 (a) Ribonucleic acid from the leaves of control plants and independent transformants for the *Gmglnβ₁* gene with and without its 3' UTR (GSβ₁53 and GSβ₁50, respectively) were subjected to Northern blot hybridization using *Gmglnβ₁* cDNA as a probe. The ethidium bromide stained pattern of the rRNA is shown as an indicator of RNA loads. Sample numbers indicate the individual control and transgenic lines.
 (b) Protein extracts (5 μg for SDS-PAGE and 10 μg for native gels) from leaves of control and transformed plants were subjected to SDS-PAGE (SDS) and native (Native) gel electrophoresis followed by immunoblotting using anti-GS antibodies. The bands for GS₂ and GS₁ are indicated. The octameric (8mer) and tetrameric (4mer) forms of GS were identified according to their *M_r* by comparison to standards included in the gel (not shown).
 (c) GS activity was measured in the leaves of several control and independent transformed plants by the transferase activity assay. Values represent the average ± SD.

Figure 9. Northern blot analysis of transgenic tobacco plants transformed with a reporter gene construct containing the 3' UTR of the *Gm glnβ₁* gene

(a) The gene construct composed of the CaMV35S promoter, the β-glucuronidase (*uidA*) reporter gene and the NOS terminator within the plasmid pBI 121 was engineered to include the 3' UTR of the *Gm glnβ₁* gene downstream of the reporter gene (*uidA*::*glnβ₁* 3' UTR). The polyadenylation sites in the *Gm glnβ₁* and the NOS 3' UTRs are indicated by asterisks (*).

(b) Ribonucleic acid was isolated from control non-transformed plants and plants transformed with the two gene constructs, and analyzed by Northern blot using a ³²P-labeled probe corresponding to the *uidA* gene. The SYBR Gold stained gel is shown as a loading control.

Figure 10. Model for regulation of GS₁ in plants

The model includes mechanisms and factors that are important in the expression and regulation of GS₁ in plants. The 3' UTR mediated mechanisms that play a role in transcript turnover and translation inhibition are new additions to this pathway and are based on the experimental data presented here.