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The 5' untranslated region of the soybean cytosolic glutamine synthetase β_1 gene contains prokaryotic translation initiation signals and acts as a translational enhancer in plants

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Abstract

Glutamine synthetase (GS) catalyzes the synthesis of glutamine from glutamate and ammonia. In plants, it occurs as two major isoforms, a cytosolic form (GS₁) and a nuclear encoded chloroplastic form. The focus of this paper is to determine the role of the 5'UTR of a GS₁ gene. GS₁ gene constructs with and without its 5' and 3' UTRs, driven by a constitutive promoter, were agroinfiltrated into tobacco leaves and the tissues were analyzed for both transgene transcript and protein accumulation. The constructs were also tested in an in vitro transcription/translation system and in *Escherichia coli*. Our results showed that while the 3' UTR functioned in the destabilization of the transcript, the 5' UTR acted as a translation enhancer in plant cells but not in the in vitro translation system. The 5'UTR of the GS₁ gene when placed in front of a reporter gene (*uidA*), showed a 20-fold increase in the level of GUS expression in agroinfiltrated leaves when compared to the same gene construct without the 5'UTR. The 5'UTR-mediated translational enhancement is probably another step in the regulation of GS in plants. The presence of the GS₁ 5' UTR in front of the GS₁ coding region allowed for its translation in *E. coli* suggesting the commonality of the translation initiation mechanism for this gene between plants and bacteria.

Keywords

Post-transcriptional regulation; Translational control; Glutamine synthetase; β -Glucuronidase; Agroinfiltration

Introduction

Plant glutamine synthetase (GS) occurs as two different isoforms, encoded by nuclear genes but located either in the cytosol (GS₁) or in the chloroplast (GS₂). Glutamine synthetase (GS) plays an important role in nitrogen metabolism in plants since it functions in the ATP-dependent conversion of an inorganic form of nitrogen (ammonia) to an organic form (glutamine).

The synthesis of glutamine is the first step for the synthesis of all other essential nitrogenous compounds contained in the cells. GS also plays a crucial role in removing ammonia which is toxic to the cells, but at the same time GS levels and activity have to be fine-tuned to maintain a balance between the rates of amino acid biosynthesis and the cellular C skeletons and ATP levels, that could be depleted by GS activity (Nunes-Nesi et al. 2010). GS in bacteria is highly regulated in vivo by transcriptional and post-translational mechanisms including adenylation and metabolic feedback inhibition (Leigh and Dodsworth 2007). The glutamine/ α -ketoglutarate ratio and the adenylation energy charge are critical in the control of GS activity in bacteria (Ninfa and Jiang 2005; Jiang and Ninfa 2007). GS in plants, as in bacteria, is also regulated at multiple levels (Ortega et al. 2006; Bernard and Habash 2009). It has been proposed that the glutamine/ α -ketoglutarate ratio may also be important for the control of nitrogen assimilation in plants (Smith et al. 2003; Nunes-Nesi et al. 2010). We have found that regulation of expression of a soybean (*Glycine max*) GS₁ gene (*GmglN β ₁*) driven by a constitutive promoter (CaMV 35S) in transgenic plants occurs at a post-transcriptional level (Ortega et al. 2001, 2006). We have also shown that the post-transcriptional regulatory step of the *GmglN β ₁* and the GS₁ genes from alfalfa is, at the level of transcript turnover, mediated by their 3'UTR (Ortega et al. 2006; Simon and Sengupta-Gopalan 2010). The goal of this study was to investigate if *GmglN β ₁* is also subject to other modes of regulation by testing the role of its 5'UTR in the regulation of gene expression.

Analysis of the translation process of eukaryotic mRNAs has shown that the 5'UTR plays an important role in translation initiation by its secondary structure; the context of AUG codon; and the existence of AUG or upstream open-reading frames (Kawaguchi and Bailey-Serres 2005). The 5'UTR is also the target for the binding of microRNAs that cause translational repression (Grey et al. 2010) or enhance translation (Orom et al. 2008). There are also some reports of the 5'UTR having a role in mRNA stabilization, though this is usually an attribute of sequences in the 3'UTR (Zou et al. 2003).

To demonstrate the role of the 5'UTR of the *GmglN β ₁* gene in the regulation of its expression, a series of gene constructs were made with the *GmglN β ₁* driven by the CaMV 35S promoter and tested for their expression by agroinfiltration in tobacco (*Nicotiana tabacum* cv. *xanthi*). Leaves were infiltrated with the *GmglN β ₁* gene constructs containing its own 5' and 3' UTRs or the corresponding gene fusions in which the GS₁ 5'UTR was replaced with the 5' noncoding region of the *uidA* reporter gene of the CAMBIA 2301 vector (Roberts et al. 1998) or the 3' UTR replaced by the NOS 3' end (Rogers et al. 1987). Transient expression through agroinfiltration is a relatively easy procedure known to be effective in analyzing expression of transgenes (Leckie and Stewart 2011). Our results showed that whereas the 3'UTR of the *GmglN β ₁* gene is involved in the control of the mRNA stability, the *GmglN β ₁* 5' UTR enhances the translation of both the GS₁ gene and a β -glucuronidase (*uidA*) reporter gene in plants.

For the complete characterization of the role of the 5' UTR of the *GmglN β ₁* gene as a translation enhancer, we also addressed the question of whether the 5'UTR of *GmglN β ₁* would have the necessary information to allow initiation of translation in a bacterial cell. In the traditional view of translation initiation, there are major differences between eukaryotes and prokaryotes in the way the ribosomes are recruited to the mRNA and this is mediated by sequences in the 5' noncoding region. However, there are reports that eukaryotic ribosomes can recognize prokaryotic signals and initiate synthesis at internal sites of polycistronic mRNAs (Nakamoto 2009). Similarly, the *E. coli* ribosomes have been shown to recognize eukaryotic viral initiation signals and translate eukaryotic viral mRNAs suggesting that translation initiation signals in prokaryotes and eukaryotes are similar (Nakamoto 2011). To corroborate the universality of the translation initiation mechanism between prokaryotes and eukaryotes, we introduced the *GmglN β ₁* gene with its 5'UTR in *E. coli* and showed

accumulation of the corresponding protein in the bacterial cells, thus supporting the postulate that the mechanisms of translation initiation for this gene are conserved between plants and bacteria and may further support the notion that GS genes originated from a gene duplication event that preceded the divergence of prokaryotes and eukaryotes (Kumada et al. 1993).

Materials and methods

Biological material

Agrobacterium tumefaciens strain GV3101 (*Rif^r*) was used for tobacco infiltration experiments. Tobacco plants (*Nicotiana tabacum* var. *xanthi*) were propagated from cuttings of tops of plants grown under sterile conditions in magenta boxes containing solid Murashige and Skoog media supplemented with 1 % sucrose. New tops were cut and transferred to new media every 3 weeks. Plant tops with developed roots were moved to pots with sterile vermiculite and grown for 3 weeks at a 16 h light/8 h dark cycle under fluorescent light before infiltration with *Agrobacterium*. Plants were maintained with Hoagland's nutrient solution containing 5 mM NH_4NO_3 .

Gene constructs

The isolation of the *Gmgl β ₁* gene (NCBI accession number AF301590) was previously described (Ortega et al. 2001). Four different GS β ₁ gene constructs with/without its 5' and 3'UTRs were made. The *Gmgl β ₁* gene constructs (Fig. 1) either included its own 5'UTR (GS β ₁₅₀, GS β ₁₅₃), its own 3'UTR (GS β ₁₀₃, GS β ₁₅₃), or lacked both its 5' and 3'UTR (GS β ₁₀₀). In GS β ₁₅₀ and GS β ₁₀₀, the 3'UTR was replaced with the NOS 3'UTR and in pGS β ₁₀₃ and GS β ₁₀₀ the 5'UTR was replaced with the mRNA leader of the CaMV35S promoter. *uidA* gene construct in the CAMBIA 2301 plasmid (CAMBIA, Canberra, Australia). To make the gene constructs with the *Gmgl β ₁* 5'UTR, the CaMV35S promoter was amplified, using pCAMBIA2301 plasmid as a template, with reverse primers with an *EcoRI* site downstream of the CaMV35S transcription start site (5'-GAG AAT TCC CGT GTT CTC TCC AAA TGA AAT GAA CTT C-3'). The CaMV35S promoter for the *Gmgl β ₁* gene constructs without the 5'UTR was amplified with a reverse primer that included extra nucleotides encoding the first seven amino acids of the *Gmgl β ₁* gene, down to a *BglIII* site (5'-AGA TCT GAG AGC GA GAC ATG GTC AAG AGT CCC CCG TGT T-3'), which was used to clone the remaining of the *Gmgl β ₁* gene (Fig. 1b). The forward primer for amplification of the CaMV35S promoter included a *HindIII* at the 5' end (5'-AAG CTT CAT GGA GTC AAA GAT TC-3'). The *Gmgl β ₁* gene constructs without its 3' UTR (GS β ₁₅₀ and GS β ₁₀₀) were made by removing the *Gmgl β ₁* 3' UTR as previously described (Ortega et al. 2006) and replacing it with a *SmaI/PstI* NOS 3' UTR fragment amplified from the pMON316 vector (forward primer: 5'-CCC GGG ATC GTT CAA ACA TTT GGC AAT AAA GTT-3'; reverse primer: 5'-GAG CTC TGC AGC CCG ATC TAG TAA CAT AGA CAC C-3'). For the β -glucuronidase gene construct GUSA50, the CaMV35S promoter containing the *Gmgl β ₁* 5'UTR was amplified from the GS β ₁₅₃ gene construct by PCR using a primer containing a *NcoI* site at the translation start site (Fig. 4, reverse primer: 5'-CCA TGG CTC CAA AAT CAA GCA AAG ACC CTT CTT T-3'), which was then ligated to the *uidA* gene excised from the CAMBIA 2301 plasmid as a *NcoI/BstEII* fragment. A *BstEII/PstI* NOS 3'UTR fragment was amplified by PCR from the CAMBIA 2301 (forward primer: 5'-GGT GAC CAG CTC GAA TTT CCC CGA TCG TTC AAA CAT TTG GC-3') and a NOS *SacI/PstI* reverse primer (as above) and placed at the end of the *uidA* gene. All PCR products were cloned into the pGEM-T easy vector (Promega, Madison WI) and sequenced. Gene constructs were assembled in the pBluescript II KS (Ⓜ) plasmid. The constructs were sequenced and subsequently cloned in the

pCAMBIA 2300 vector as *KpnI/PstI* inserts and transformed into *A. tumefaciens* strain GV3101 for plant infiltration.

To test the role of the *Gmglb β ₁* 5'UTR in bacterial translation, a dicistronic gene construct, plasmid pGEX: :*GS153*, was produced. The plasmid pGEX: :*GS153* has the full length *Gmglb β ₁* cDNA in the glutathione transferase expression plasmid pGEX-4T-2 (GE Healthcare) and was created by inserting the *Gmglb β ₁* cDNA as a transcriptional fusion in the *EcoRI* fragment at the end of the GST gene. A dicistronic plasmid containing the *Gmglb β ₁* gene with its 5' UTR replaced by 5' mRNA leader of the CAMBIA2301 vector was used as control for expression in *E. coli*. This plasmid, pGEX: :*GS103*, was made by inserting a *GS₁* RT-PCR product, amplified from tobacco infiltrated plants as described below, as an *EcoRI* fragment in the pGEX-4T-2 vector. Both the 5'UTR of the *Gmglb β ₁* gene and the mRNA leader of the CAMBIA reporter gene introduced a stop codon, producing a GST protein in which the last nine amino acids were replaced by either a leucine, in the plasmid pGEX: :*GS153*, or the amino acids arginine and proline in the plasmid pGEX: :*GS103* (Fig. 7b).

Agroinfiltration of tobacco leaves

Infiltration of *N. tabaccum xanthi* var. *xanthi* leaves was done as a modification of the method described by Koscińska et al. (2005) and Wang et al. (2010). Single colonies of *A. tumefaciens* containing the different gene constructs were grown overnight in LB media with kanamycin and rifampicin selection (50 $\mu\text{g ml}^{-1}$ each). Cultures were diluted tenfold in LB media and grown with antibiotic selection to a 0.5 OD₆₀₀. Cells were centrifuged and resuspended in the same volume of induction media (10 mM MES, 10 mM MgCl₂, 0.2 mM acetosyringone, pH 5.7) and incubated overnight at 30 °C with shaking. *Agrobacterium* cells were then centrifuged and resuspended in the same volume of infiltration media (1 mM MES, 0.5 mM MgCl₂, 0.02 mM acetosyringone, pH 5.7). The three youngest leaves of each tobacco plant were uniformly infiltrated with the *Agrobacterium* suspension (two plants per gene construct) using a 1-ml disposable syringe without a needle. Four days after infiltration, the middle vein of the leaf was removed and the leaf blades were used for protein extraction or harvested in liquid nitrogen and frozen at -80 °C for RNA isolation. All experiments were repeated at least three times. Data presented are the results of at least three independent experiments.

Isolation and analysis of RNA

RNA was isolated from leaf tissues by phenol/chloroform extraction and LiCl precipitation as previously described (Ortega et al. 2001). Total leaf RNA was subjected to 1.5 % (w/v) formaldehyde-agarose gel electrophoresis followed by northern blotting using ³²P-labeled DNA probes as described in figure legends. The same blot was hybridized to the different probes following stripping before hybridizing with the next probe.

RNA secondary structure of the *Gmglb β ₁* 5'UTR was predicted with the RNA folding by free energy minimization software MFOLD 2.0 (Jaeger et al. 1989; Zuker 1989) and with the Unified Nucleic Acid Folding software UNAFold 3.8 (Markham and Zuker 2008) with similar results.

In vitro transcription and translation

Total RNA isolated from tobacco leaves infiltrated with the different *GS₁* gene constructs was reverse transcribed (RT) with an oligo-dT containing an anchor primer (5'-CCA GTG AGC AGA GTG ACG AGG AAG CTT₍₁₇₎-3'). *GS₁* PCR products were further amplified from the cDNA with a forward primer for the *GS₁* 5'UTR (5'-CAC GGG AAT TCT CTA AAA GAG ATC TT-3' for constructs *GS β 150* and *GS β 153*), or with a forward primer for

the CaMV35S mRNA leader (5'-AAC ACG GGG GAC TCT TGA CCA T-3' for constructs GS β 100 and GS β 103), and a reverse primer corresponding to the anchor sequence, which contained a *Hin*-dIII site at the end to preserve a continuous polyA tail. GS₁ PCR products were cloned in pCRBlunt II (Life Technologies, Carlsbad, CA) and sequenced. RNA was in vitro transcribed from the RT-GS β ₁ clones with a T7 RNA synthesis kit (Life Technologies) and purified. Equal amounts of capped and polyadenylated RNA were translated in vitro in a wheat germ system (Promega, Madison, WI) and the products were analyzed by Western blotting using GS₁ antibodies.

Protein isolation and analysis

Fresh leaf tissue was ground in a mortar with 2 volumes of extraction buffer (25 mM Tris, 20 % glycerol, 5 % ethylene glycol, 1 mM MgCl₂, 1 mM EDTA, 0.1 % Triton X100, 5 mM DTT, 3 % insoluble PVPP, pH 8). Expression of the GS₁ in *E. coli* was analyzed by inducing the expression of the Gmgl β ₁ gene in the pGEX-4T-2 constructs in 10 mL bacterial cultures, grown to an exponential phase, with 5 mM IPTG for 2 h. Cells were harvested by centrifugation at 5,000×g for 5 min at 4 °C. Bacterial pellets were resuspended in 1 mL of extraction buffer. *E. coli* proteins were extracted in the same extraction buffer without PVPP; Triton X100 was added after sonication for five cycles of 10 s each. Plant and bacterial extracts were clarified by centrifugation at 20,000 × g for 15 min at 4 °C and transferred to new tubes. Protein concentration was determined colorimetrically with a protein assay solution (Biorad, Hercules, CA). Protein extracts were fractionated by non-denaturing or SDS polyacrylamide gel electrophoresis as described (Ortega et al. 2006), followed by blotting to PVDF transfer membranes (Millipore, Billerica, MA). Proteins were immunodetected with antibodies raised against soybean GS₁ or with commercially available anti- β -glucuronidase (GUS) antibody (ABCAM, Cambridge, MA) and anti-glutathione transferase (GST) antibody (GE Healthcare, Piscataway, NJ) and a secondary antibody coupled to alkaline phosphatase. GS immunoreacting bands were quantified using Carestream Molecular Imaging Software 5.0 (Carestream Health, Rochester, NY). An unpaired *t* test statistical analysis of the GS band intensities was performed using Prism 5.0 software (GraphPad Software).

Measurement of glucuronidase (GUS) activity

β -Glucuronidase activity in extracts of infiltrated tobacco plants was determined using the fluorogenic substrate 4-methyl umbelliferyl glucuronide (4-MUG, Gold Biotechnology, Saint Louis, MO), as described in Jefferson et al. (1987). Leaf extracts were incubated in a 1-mL reaction containing 1 mM 4-MUG at 37 °C. Aliquots (200 μ L) were removed at 10 or 30 min intervals, the reaction was stopped by adding 1.8 mL of 0.2 M Na₂CO₃ and the fluorescence of 4-methyl umbelliferone (4-MU) produced was measured in a fluorometer (Turner Designs, Sunnyvale, CA) with an excitation wavelength at 365 nm, emission filter at 455 nm. GUS-specific activity was calculated against a 4-MU standard curve (from 0.1 to 5 μ M).

Results

The 5'UTR Gmgl β ₁ gene does not affect the accumulation of mRNA in infiltrated tissues

To check if the 5'UTR of a cytosolic GS gene has any role in regulating transcript stability or translation, gene constructs driven by the CaMV 35S promoter consisting of a Gmgl β ₁ coding region containing or lacking its own 3'UTR and 5'UTR (Fig. 1) were examined by transient expression assays by means of *A. tumefaciens* infiltration of tobacco leaves (*Nicotiana tabacum* var. *xanthi*). Four days after infiltration, the accumulation of the Gmgl β ₁ mRNA was analyzed (Fig. 2). RNA isolated from the infiltrated leaves was subjected serially to northern blot analysis using the coding region of the Gmgl β ₁ gene, the

3'UTR of *Gmgl β ₁* gene, the NOS terminator and the NPTII gene as probes. Control tobacco plants infiltrated with *A. tumefaciens* without any plasmid did not show hybridization to any of the DNA probes. The accumulation of the NPTII mRNA was monitored to determine the efficacy of the infiltration procedure and to standardize for the *Gmgl β ₁* mRNA accumulation levels. As seen in Fig. 2, the level of hybridization in the leaf samples infiltrated with the different gene constructs showed some differences in hybridization with the NPTII probe, probably an attribute of the uniformity of the agroinfiltration, i.e. the number of cells that get the T-DNA during the process rather than position effect. The *Gmgl β ₁* coding region showed hybridization to all the samples infiltrated with the GS₁ gene constructs, although to different levels (Fig. 2). Quantification and standardization of the mRNA band intensities (Fig. 2b) showed that the *Gmgl β ₁* mRNA accumulation in leaves infiltrated with the gene constructs containing the 3' UTR (GS β 103 and GS β 153) was ten times lower compared to the mRNA accumulation levels of the *Gmgl β ₁* gene with the NOS 3' UTR (GS β 150, GS β 100). This indicates that the 3'UTR of the *Gmgl β ₁* gene downregulates mRNA levels at a post-transcriptional step, as has been demonstrated in stably transformed alfalfa and tobacco plants (Ortega et al. 2006). As is also seen in Fig. 2, there is no difference in the transcript level for *Gmgl β ₁* gene in tissues infiltrated with the *Gmgl β ₁* gene constructs containing its own 5'UTR compared to the corresponding gene fusions in which the GS₁ 5'UTR was replaced with the 5' noncoding region of the *uidA* reporter gene of the CAMBIA 2301 (GS β 150 compared to GS β 100 and GS β 153 compared to GS β 103, Fig. 1). The 3'UTR of the *Gmgl β ₁* gene and the NOS terminator were used as probes to further distinguish between the transcript levels for the *Gmgl β ₁* gene in the different constructs. Hybridization with the 3' UTR and the NOS terminator as probes showed no significant difference in the intensity of the hybridization signal between the constructs with and without the 5'UTR, GS β 103 against GS β 153 and GS β 100 against GS β 150, respectively. These results indicate that the *Gmgl β ₁* 5' UTR does not have a role in controlling GS₁ transcript levels and that the 3'UTR functions independently of the 5'UTR, in controlling transcript turnover.

The inclusion of the *Gmgl β ₁* 5'UTR in the gene constructs enhances the accumulation of GS₁ protein in the infiltrated tissues

To check if the 5'UTR of the GS₁ gene affects translation, we analyzed the pattern of GS protein accumulation and GS holoenzymes in tobacco leaves infiltrated with the different CaMV35S: *Gmgl β ₁* gene constructs in protein blots with antibodies against the soybean GS β ₁ protein. GS is an octameric enzyme and can be either homomeric or heteromeric; based on its composition GS shows slight differences in the migration pattern on native gels. Control tobacco leaf GS₁, in its native conformation migrated as a complex mixture of isoforms, probably representing different heteromeric forms (Fig. 3a). *Gmgl β ₁*-infiltrated samples showed another immunoreactive band in addition to the endogenous complexes seen in control samples, the level being relatively higher in the GS β 100 and GS β 150-infiltrated tissues compared to GS β 153-infiltrated tissues, in keeping with the corresponding transcript level (Fig. 2). However, GS β 103 infiltrated samples showed a GS₁ holoenzyme profile similar to that seen in control samples, because of the low level of expression of this gene construct. To look for quantitative differences in the steady state level of the *Gmgl β ₁* protein with the different gene constructs, the infiltrated tissue extracts were subjected to SDS PAGE followed by Western blot analysis. As seen in Fig. 3, the *Gmgl β ₁* protein co-migrated with the endogenous tobacco GS₁ in their denatured form. The GS₁ immunoreactive band was quantified and the intensity was standardized against the average intensity of the GS β 153 gene construct, which contains the unmodified *Gmgl β ₁* gene that includes its own 5'- and 3'-UTRs (Fig. 3b). The accumulation levels of the GS₁ protein in the tissues infiltrated with gene constructs that lack the *Gmgl β ₁* 3'UTR (GS β 100, GS β 150) was higher than the accumulation of GS₁ in tissues infiltrated with the *Gmgl β ₁* constructs

containing the *Gmgl β ₁* 3' UTR (GS β 103, GS β 153), in keeping with the corresponding transgene transcript level (Fig. 2). However, in spite of the fact that tissues infiltrated with GS β 150 and GS β 100 showed the same level of the transgene transcript, the GS₁ accumulation levels showed differences that were statistically significant. The GS β 150 infiltrated tissues showed a 27 % higher level of GS₁ accumulation than GS β 100 infiltrated tissues. Similarly, the GS β 153 infiltrated tissues showed a significant 31 % higher level of GS₁ protein accumulation compared to GS β 103 infiltrated tissues, even though there was no difference in the level of transgene transcript accumulation. The GS₂ protein levels in the denatured samples showed a reduction in the transformants compared to control plants. The same results were obtained in multiple experiments. The difference in the steady state level of the GS₁ protein between tissues infiltrated with *Gmgl β ₁* gene constructs with or without its 5'UTR cannot be attributed to differences in transcript level (Fig. 2) or to differential protein turnover since the coding sequence and hence the amino acid sequence is identical between the gene constructs. These results thus suggest that the 5'UTR plays a role in enhancing translation and that it functions independent of the 3'UTR.

***Gmgl β ₁* 5'-UTR enhances the translational capacity of the *uidA* transcript**

To further demonstrate that the *Gmgl β ₁* 5' -UTR has a role in enhancing translation, a gene construct was made with the 5'UTR of the *Gmgl β ₁* gene inserted between the CaMV 35S promoter and the β -glucuronidase reporter gene (*uidA*). The resulting gene construct GUSA50 and the parent CAMBIA 2301 plasmid, which consists of the *uidA* reporter gene driven by the CaMV 35S promoter (Fig. 4a), were agroinfiltrated into tobacco leaves. Protein was extracted 4 days after infiltration and analyzed by Western blotting with anti- β -glucuronidase (GUS) antibodies and measurement of its enzymatic activity (GUS activity). An immunoreactive band of 71 kDa, corresponding to the predicted size of the *uidA* gene product, accumulates to a more than tenfold higher level in the tissues infiltrated with GUSA50 than in the CAMBIA 2301 samples (Fig. 4b), while the control samples infiltrated with the empty binary plasmid, CAMBIA 2300, did not show the corresponding band. GUS activity measurements showed that the inclusion of the *Gmgl β ₁* 5'UTR in the 35SCaMV: *uidA* reporter gene construct increased the expression of GUS by 22.8-fold compared to the GUS expression in plants infiltrated with the original CAMBIA 2301 plasmid (Fig. 4c). The results suggest that the 5'UTR of the *Gmgl β ₁* gene functions as a translation enhancer independent of the coding region and the 3'UTR of the *Gmgl β ₁* gene.

The 5'UTR-mediated translation enhancement does not occur in the in vitro translation systems

To check if translation enhancement by the *Gmgl β ₁* 5'UTR was due to its interaction with some factor that is only present in the plant cells, we used the in vitro reticulocyte and the wheat germ translation systems to check for translational regulation of the *Gmgl β ₁* transcript with or without its 3' and 5'UTRs. Total RNA from tobacco leaves agroinfiltrated with the different gene constructs was reverse transcribed with an oligo-dT containing an anchor primer, and PCR amplified with a forward primer for the *Gmgl β ₁* 5'UTR (for constructs GS β 150 and GS β 153) or with a forward primer for the CaMV35S mRNA leader (constructs GS β 100 and GS β 103) and a reverse primer corresponding to an anchor sequence which contained a *Hind*III site to preserve a continuous polyA tail. *Gmgl β ₁* PCR products were cloned and sequenced. RNA was then transcribed in vitro from the RT-GS β ₁ clones and analyzed by subjecting equal amounts of the reaction products to electrophoresis followed by nucleic acid staining. No difference was observed in the transcript level for the different gene constructs (data not shown) suggesting that the 3'UTR-mediated transcript turnover does not take place in the in vitro system. Equal amounts of capped and polyadenylated RNA were then translated in a rabbit reticulocyte system and in a wheat germ system and equal amounts of the translation reaction products were analyzed by Western

blotting using an anti-GS₁ antibody. No difference in the accumulation of the GS₁ protein band translated from the different GS₁ transcripts was seen either in the rabbit reticulocyte system (data not shown) or in the wheat germ system (Fig. 5), indicating that the Gmgl β ₁ 5' UTR did not enhance translation in the in vitro system and nor did the 3'UTR had any effect on translation rates in the in vitro translation system.

Sequence analysis of the 5'UTR of the Gmgl β ₁ gene shows some unique features

Because of the role that the 5'UTR of the Gmgl β ₁ gene plays in translation, the 5'UTR was sequenced and analyzed for any distinguishing features. The minimum energy folding of the Gmgl β ₁ 5'UTR shows a hairpin structure with the AUG start codon in an open unfolded region (Fig. 6). We also found a few features that are usually found in the noncoding regions of bacterial mRNA, like a track of purines at position -20 to -32 relative to the start codon that has complementarity to the 16S rRNA in its mRNA 'track' region (Fig. 7b). There is a partial Shine-Dalgarno (SD) consensus sequence (GGAG) in the Gmgl β ₁ gene at position -1 relative to the start codon. Immediately upstream of the track of purines in the Gmgl β ₁ 5'UTR, there is a sequence similar to the *E. coli* *rrnB* BoxA, which though a transcriptional antiterminator, binds the ribosomal S1 protein and acts as a translational enhancer in *E. coli* (Mogridge and Greenblatt 1998). It has been shown that S1 is the major component of the *E. coli* ribosome directly involved in mRNA selection (Komarova et al. 2002). We, therefore, proposed that the Gmgl β ₁ gene transcript could be translated in the bacterial cells.

The 5' UTR of the Gmgl β ₁ gene allows its translation in *E. coli*

To check our postulate that the regions similar to bacterial translational elements, found in the 5'UTR of the Gmgl β ₁ gene transcript, have the potential to recruit bacterial ribosomes, we analyzed the expression in *E. coli* of the plasmid pGEX: :GS153, which contains a transcriptional fusion of the Gmgl β ₁ gene downstream of glutathione transferase (GST) gene in the expression vector pGEX-4T-2 (GE Healthcare). The two genes would be transcribed from the IPTG-inducible *P tac* promoter resulting in a dicistronic mRNA encoding a GST protein followed by the gln β ₁ 5'UTR that would direct the translation of the GS β ₁ protein. Sequence analysis showed no indication of a translational fusion of the two genes (Fig. 7). Another GST: :gln β ₁ gene construct was made, plasmid pGEX: :GS103, with the pCAMBIA2301 mRNA leader replacing the 5'UTR of the Gmgl β ₁ gene. The expression of the GST and Gmgl β ₁ genes in the dicistronic plasmids was induced by IPTG in exponentially growing *E. coli* cultures. Proteins were extracted and subjected to Western blot analysis using anti-GS and anti-GST antibodies. As seen in Fig. 7, both the GST and the GS β ₁ proteins accumulate in the bacteria expressing both gene constructs. The dicistronic plasmids expressed a protein that co-migrated with the GS₁ band from a tobacco leaf sample over-expressing the Gmgl β ₁ gene. The CAMBIA mRNA leader has translation initiation signals for both prokaryotic and eukaryotic cells (Roberts et al. 1998) and thus it was expected that the Gmgl β ₁ with the CAMBIA leader would be translated in *E. coli*. Similarly, the expression of Gmgl β ₁ in the pGEX in *E. coli* cells confirms that the Gmgl β ₁ 5'UTR is functional in recruiting ribosomes in a manner similar to an internal ribosomal entry site (IRES), allowing the expression of the Gmgl β ₁ gene. The level of accumulation of the GS β ₁ protein in *E. coli* with the GST: :GS103 gene construct was ~threefold higher than the level accumulating with the pGEX: :GS153 construct. The expression of the GST protein in the GST: :gln β ₁ gene constructs is not affected, except for the fact that the GST protein is eight and nine amino acids shorter in the GS103 and GS153 gene fusions, respectively (Fig. 7). To the best of our knowledge, this is the first demonstration of the 5'UTR of an eukaryotic gene functioning as an IRES in *E. coli*.

Discussion

The data presented here, points to the 5' UTR of the *Gmgl β 1* gene as the determinant of translation enhancement and confirms our earlier finding that the 3'UTR of the *Gmgl β 1* gene controls the turnover of the transcript (Ortega et al. 2006; Simon and Sengupta-Gopalan 2010). In this study, a transient assay using agroinfiltration has allowed us to simultaneously check the expression of different gene constructs involving the 5' and 3'UTRs of the *Gmgl β 1* gene at the level of transcript and protein accumulation. By testing gene constructs in different combinations, with/without the 3'UTR/5'UTR, we have demonstrated the role of the 5'UTR of the *Gmgl β 1* gene in translational control and the 3'UTR in transcript turnover with both the UTRs functioning independent of each other.

Northern blot analysis using different probes, showed a 20-fold higher level of accumulation of the *Gmgl β 1* transcript in the tissues infiltrated with the gene with the NOS terminator (pGS β 100 and pGS β 150) compared to tissues infiltrated with the *Gmgl β 1* gene constructs containing its own 3'UTR (pGS β 103 and pGS β 153; Fig. 2). No differences in transcript levels were found between the samples with the gene constructs with the *Gmgl β 1* 5' UTR compared to the samples with the gene constructs without the 5'UTR when hybridized with either the GS $_1$ -coding region or the 3'-specific regions. We thus concluded that the 5'UTR of the *Gmgl β 1* did not play a role in the 3'UTR-mediated turnover of the GS $_1$ transcript.

Comparison of the amounts of GS $_1$ protein in the infiltrated tissues showed a significantly higher level of the GS $_1$ polypeptide, 2.5-fold the amount of the endogenous GS $_1$ levels, in the tissues infiltrated with the pGS β 150 gene construct (Fig. 3). The pGS β 150 gene construct showed 27 % higher GS $_1$ protein accumulation than with the *Gmgl β 1* gene without its 5'UTR (pGS β 100). Similarly, the pGS β 153-infiltrated tissues showed a higher level of GS $_1$ polypeptide compared to the pGS β 103 samples, with no difference in the corresponding transgene transcript level. Besides its own coding region and 3'UTR, the 5'UTR of the *Gmgl β 1* transcript when placed in front of the *uidA* gene showed a huge increase in translation of the reporter gene, further emphasizing the role of *Gmgl β 1* gene in enhancing translation. Some studies have indicated that the 5'UTR-mediated translation enhancement requires the interactions between the 5' and 3'UTRs of the messenger RNA (Kawaguchi and Bailey-Serres 2002; Nagaya et al. 2010; Stupina et al. 2011). In these studies it has been shown that the 5' and 3' ends of an mRNA interact via protein-RNA contacts between the 5'cap or 5'UTR and the 3'UTR or the 3'poly(A)-tail. Proteins involved in the interaction could be translation factors, poly(A)-binding proteins or specific mRNA-binding proteins; such interactions may not be absolutely required for translation but may improve translational efficiency. Our results support the notion that the 5'UTR of the *Gmgl β 1* gene functions as a translation enhancer. The 5'UTR-mediated translational enhancement has been shown for other genes in the literature including the genes for alcohol dehydrogenase, a fatty acid desaturase, genes for heat shock proteins and a pollen gene, *ntp303* (Dansako et al. 2003; Hulzink et al. 2002; Satoh et al. 2004; Wang and Xu 2010). The 5'UTR of the *Gmgl β 1* gene functions in an autonomous manner and functions as a translation enhancer independent of the coding region and the 3' UTR of the *Gmgl β 1* gene. The 20-fold increase in expression level of the *uidA* gene with the 5'UTR of the *Gmgl β 1* gene compared to the expression level of the *uidA* gene with its own mRNA leader (Fig. 4), is striking when compared to the expression of the *Gmgl β 1* gene with its 5'UTR which accounted for only 27–31 % increase in total GS $_1$ protein accumulation, compared to the gene constructs without the 5'UTR. The difference could be attributed to endogenous control of the GS $_1$ levels or to sequences in the GS $_1$ -coding region controlling the initiation and elongation steps of translation.

The translation efficiency of mRNA could be affected by the stability of the 5'UTR. The stem-loop region of the tobacco *psbA*-5'UTR and the Omega leader of the TMV RNA are important determinants of mRNA translation that act as translation enhancers because of their stable secondary and tertiary structure (Zou et al. 2003; Agalarov et al. 2011). The predicted secondary structure of the 5'UTR of the *Gmglb β ₁* is a stable hairpin with a free energy of $-27.1 \text{ kcal mol}^{-1}$ that could play a role in translation enhancement (Fig. 6).

The 5'UTR of the *Gmglb β ₁* gene does not function as a translation enhancer in an in vitro translation system (Fig. 5). This would imply that some kind of factors, not present in the cell-free systems but present in the leaf cells, interact with the 5' UTR of *Gmglb β ₁* gene to enhance translation rates ruling out the possibility of the 5' UTR acting as translational enhancer just based on its secondary structure. Such factors may be synthesized or post-translationally modified under certain physiological conditions. The interaction between *trans*-factors and *cis*-acting elements in the 5'UTR of some genes, has been shown to play a role in translational enhancement. Glucose-mediated translational enhancement of animal mRNAs in the pancreas is due to the binding of specific factors to the 5' UTR (Kulkarni et al. 2011). The plant heat shock protein, HSP 101, enhances the translational activity by binding the 5'UTR of some transcripts (Gallie 2002; Kawaguchi and Bailey-Serres 2002). It has also been proposed that interactions with specific factors determine the 5'UTR-mediated enhancement of translation of the *ntp303* transcript during pollen tube growth (Hulzink et al. 2002). It would be interesting to check if the 5'UTR-mediated translational regulation of the *Gmglb β ₁* gene is tissue-specific or is dictated by the metabolic state of the tissue.

Sequence analysis of the 5'UTR of the *Gmglb β ₁* mRNA showed a track of pyrimidines at -48 to -33 nucleotides upstream of the translation start codon (Fig. 1b). 5'-Terminal oligopyrimidine tracks at the transcriptional start site (5'-TOP) are translational enhancers found in many mRNAs in animals and plants (Hamilton et al. 2006; Ma and Blenis 2009; Tzeng et al. 2009). Proteins encoded by some of the known 5'-TOP RNAs are involved in cell division and are regulated by the TOR-S6 kinase signaling pathway. Sequence analysis has shown that the plant genes involved in development, also encode 5' TOP-mRNAs (Tzeng et al. 2009). The TOR-S6 kinase signaling pathway was shown to regulate TOP-mRNAs in *Arabidopsis* plants containing a functional gene for $p70^{\text{S6k}}$, a key enzyme in this signaling pathway (Tzeng et al. 2009). The ribosomal protein S6 (RPS6) located in the head of the cytosolic 40S ribosomal subunit can interact with mRNAs, tRNAs, translation initiation factors and the 28S rRNA. It has also been found that the 40S ribosomal protein binds to the 5'UTR of the turnip crinkle virus RNA at a 19 nucleotide pyrimidine-rich element that enhances translational activity (Stupina et al. 2011). Phosphorylation of RPS6 by S6 kinases following stimulation by auxin and insulin treatments in maize leads to the translational activation of TOP-mRNAs encoding proteins associated with cell proliferation and growth (Beltrán-Peña et al. 2002; Jiménez-López et al. 2011). The presence of the oligopyrimidine track in the 5'UTR of the *Gmglb β ₁* RNA suggests that its translation may be subjected to a TOP-like translational control. This mechanism of regulating gene expression could be more widespread than suspected (Hamilton et al. 2006).

Plant glutamine synthetase genes have been expressed in *E. coli* (DasSarma et al. 1986; Bennett and Cullimore 1990; Sakakibara et al. 1996; Carvalho et al. 1997; Seabra et al. 2010). The GS₁ and GS₂ enzymes encoded by these genes are functional when expressed in bacteria, and their products correctly assemble into active native forms. However, in all cases the GS genes have been introduced as translational fusions in recombinant expression vectors. In this study, we have shown that GS₁ mRNA is efficiently translated in bacteria when introduced as non-translational gene fusions, indicating that the eukaryotic 5'UTR of the *Gmglb β ₁* mRNA contains the information to allow its translation in vivo in a prokaryotic system (Fig. 7).

Bacterial translational initiation involves the binding of the 30S ribosomal subunit to the ribosome binding site on the mRNA by base pairing between a Shine–Dalgarno (SD) region and the mRNA-binding site (MBS) at the 3' end of the 16S rRNA (McCarthy and Brimacombe 1994). There is a partial SD consensus sequence (GGAGA) in the *Gmgl β ₁* gene at position –4 to +1 relative to the start codon and a second SD region at position –25 to –20 (Fig. 1), though SD region is normally found between positions –15 to –2 relative to the start codon. Analyses of a large number of mRNA sequences have shown that base pairing is not the only significant factor in determining the efficiency of the expression of the mRNA (Lee et al. 1996). Crosslinking studies have indicated that the ribosome might interact with the mRNA via alternative sites outside the SD region and the start codon. Sequences in the 16S rRNA other than the MBS, crosslink with nucleotides both upstream and downstream of the start codon (McCarthy and Brimacombe 1994), supporting the existence of alternative mechanisms of ribosome recruitment. There is a track of purines in the *Gmgl β ₁* 5'UTR at position –20 to –33 relative to the start codon that has complementarity to the 16S rRNA in its mRNA 'track' region and may have a role in translation initiation by enhancing the efficiency of recognition of the mRNA by the ribosome. Several other mRNA *cis*-elements have a substantial effect on the translation efficiency possibly due to its involvement in the ribosome-mRNA recognition. An alternative mechanism involves *cis*-acting mRNA sequences that serve as targets for the mRNA-binding ribosomal protein S1. This protein is essential for the translational machinery of Gram negative organisms and ensures the translation of mRNAs from a wide variety of sources (Komarova et al. 2002). Immediately upstream of the track of purines in the *Gmgl β ₁* 5'UTR, there is a sequence similar to the *E. coli rrnB BoxA*, which though a transcriptional antiterminator, binds the ribosomal S1 protein and acts as a translational enhancer (Mogridge and Greenblatt 1998; Komarova et al. 2002). This region in the *Gmgl β ₁* 5'UTR may represent an efficient ribosomal S1 protein binding site. The translatability of the plant GS₁ mRNA in *E. coli* would further suggest the working of a universal mechanism for translation initiation as proposed by Nakamoto (2009, 2011). It has been proposed that the secondary structure of the 5'UTR prevents translation initiation from other sites than the initiator site, allowing for the recognition by the ribosomes via the several ligands and this mechanism is universal (Nakamoto 2009), as it seems to be the case for the GS β 1 5' UTR secondary structure which forms a stable stem structure while leaving open the initiation site (Fig. 6).

We tested the effect of the CAMBIA mRNA leader on translation of the *Gmgl β ₁* in *E. coli*, the same element was used to replace the 5'UTR of the *Gmgl β ₁* gene in plant infiltration experiments. The leader of reporter gene in the CAMBIA vectors was designed with both prokaryotic (SD) and eukaryotic (Kozak) translation initiation signals to allow strong ribosome binding and efficient translation of genes (Roberts et al. 1998). As expected, the *Gmgl β ₁* mRNA in the pGEX: :GS103 dicistronic gene construct with the CAMBIA leader is translated very efficiently in *E. coli* compared to the *Gmgl β ₁* mRNA with its own 5'UTR (Fig. 7). The difference in translation strength could be consequence of the position of the SD in the CAMBIA mRNA leader, at –15 to –10 nucleotides upstream of the start codon, that would promote optimal base pairing between the SD region and the MBS of the 16S rRNA for more efficient translation. So the conclusion that we can draw from these experiments is that the 5'UTR of *Gmgl β ₁* mRNA has sequences that allow its translation in a prokaryotic system. Whether it is a common phenomenon among other plant genes has to be tested.

Phylogenetic studies point to two classes of GS genes GSI and GSII (Kumada et al. 1993). Although there are some exceptions, GSI class is found in prokaryotes, whereas the GSII class represents the functional GS genes in eukaryotes. In contrast to the endosymbiotic hypothesis which suggests the transfer of a prokaryotic gene to the nuclei in plants during

the establishment of the endosymbiosis, the phylogenetic studies show that the GS genes involved in organelle function diverged long after the endosymbiosis event from a common ancestor GSII type gene (Kumada et al. 1993). It is thus unlikely that the remnants of translational elements in the plant GS_I that are functional in *E. coli* are of prokaryotic origin. The presence of these translation initiation elements in the GS_I rather suggests, as it has been proposed, that the mechanisms of translation initiation are conserved between prokaryotes and eukaryotes (Nakamoto 2009, 2011). It is important to determine to what extent other plant genes show conservation in their translation initiation mechanisms, particularly those genes involved in primary metabolic functions.

Work is in progress to dissect out the various components in the 5' UTR, the *trans*-factors and to determine how the different *cis*-elements/*trans*-factors function in translational regulation both in plants and in bacteria. Moreover, it is important to determine if the 5'UTR acts as a translation enhancer in a tissue-specific manner. To understand the biological significance of the translation enhancement of the GS gene by its 5'UTR, it will be important to determine if this regulatory step is mediated by signals involved in the coordination of C and N balance that control gene expression (Nunes-Nesi et al. 2010). There are reports in the literature suggesting regulation of cytosolic GS at the post-transcriptional level (Ortega et al. 2006; Simon and Sengupta-Gopalan 2010), at the posttranslational level by phosphorylation (Finnemann and Schjoerring 2000; Lima et al. 2006), through interactions with the 14-3-3 proteins (Diaz et al. 2011) and at the level of enzyme stability (Ortega et al. 1999), all steps being regulated by the N status. It is thus possible that GS_I may also be regulated at the translational level in response to the C/N status of the cells.

The ability of the Gmgl β ₁ 5'UTR to enhance translation of a protein coding region in plants is of great bio-technological significance since plant cells could be just as efficient in the synthesis of a foreign protein as bacterial cells. Moreover, plant cells would be more ideal for proteins that undergo post-translational modification. The translational enhancement property of the Gmgl β ₁ 5' UTR on the reporter gene would also be very useful for checking the functionality of weak promoters. Work is also in progress to identify the *cis*-elements in the 5'UTR of the Gmgl β ₁ gene and the *trans*-factor with which it interacts to enhance translation using stable plant transformation and agroinfiltration. Moreover, experiments will be done to test if the 5'UTR of genes encoding key enzymes in plants also have prokaryotic translation initiation sequences.

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Fig. 1.

Maps of the GS₁ gene constructs used for tobacco agroinfiltration. **a** Gene constructs consist of the soybean *Gmglβ₁* cDNA driven by the CaMV 35S promoter, either containing or lacking its 5'UTR and/or its 3'UTR. pGSβ103, is a gene construct without the 5'UTR but with the 3'UTR, pGSβ153, has both 5' and 3'UTRs, pGSβ150 has the 5'UTR but not the 3'UTR, and pGSβ100 lacks both the 5' and 3'UTRs. The *glβ₁* 5'UTR was replaced by the mRNA leader of the CAMBIA CaMV 35S promoter and the 3'UTR was replaced by the NOS terminator. **b** The nucleotide sequences of the 5'UTR of the *Gmglβ₁* and the CAMBIA leader/*Gmglβ₁* gene junctions (GSβ100 and GSβ103 gene constructs). The

translation initiation (Kozak) sequence of the CAMBIA leader is *underlined*. Nucleotides are numbered relative to the position of the translation start site, labeled with an *asterisk*. The *EcoRI* and *BglII* restriction sites used to clone the *Gmgl β ₁* gene constructs to the CaMV35S promoter are shown

Fig. 2.

Transcript analysis of *Agrobacterium*-infiltrated tobacco leaf samples. **a** For each experiment two independent plants for each construct were sampled, total RNA was isolated from 3 individual leaves from each independent plant 4 days after agroinfiltration with *A. tumefaciens* containing gene constructs of the Gm*gln β 1* gene with/ without its 5' and 3'UTRs (as described in Fig. 1). Twenty microgram of each sample was then fractionated by electrophoresis and subjected to northern blot hybridization. The blots were sequentially hybridized with 32 P labeled probes corresponding to the coding region and the 3'UTR of the Gm*gln β 1* gene, the *Agrobacterium* nopaline synthetase terminator (NOS 3'UTR) and the

kanamycin resistance (NPTII) gene. The 28S rRNA stained by SYBR Gold is also shown as a control for loading. **b** The hybridizing bands corresponding to the *Gmglu β ₁* coding region were quantified and standardized against the NPTII quantified bands and plotted. Two independent experiments were performed and similar results were obtained. Only one set of Northern blots is shown

Fig. 3.

Western blot analysis of GS protein in leaf extracts of tobacco infiltrated with *A. tumefaciens* containing *Gmglu β 1* gene constructs with/without its 5' and 3' UTRs. Proteins were extracted from tobacco leaves 4 days after infiltration with *A. tumefaciens* containing the *glu β 1* gene constructs (described in Fig. 1). For each experiment, two individual plants were agroinfiltrated with each gene construct. The proteins were fractionated by polyacrylamide gel electrophoresis in either (a) non-denaturing or (b) denaturing conditions (10 and 5 μ g, respectively), followed by immunoblotting with an antibody against the soybean GS β 1 protein. The intensities of the GS1 protein bands on the denaturing gel were

quantified and the average intensities from five independent experiments $\pm$ SD were plotted. A different *letter* (*a–d) next to a *bar* means that the differences between these samples were statistically significant ($P < 0.05$). Data shown are the results from five independent experiments. Only one representative Western blot is shown here

Fig. 4. Effect of including the 5'UTR of *Gmgl β 1* in gene constructs with *uidA* reporter gene on the expression of β -glucuronidase (GUS) in agroinfiltrated tobacco leaves. **a** Tobacco plants were infiltrated with *A. tumefaciens* containing the CAMBIA 2301 plasmid (35SCaMV promoter: *uidA* reporter gene), or with the *GUSA50* gene construct, which consists of the 5'UTR of the *Gmgl β 1* gene inserted between the CaMV 35S promoter and the *uidA* gene of the CAMBIA 2301 plasmid. **b** Four days after infiltration protein was extracted from the tobacco leaves and 50 μ g of protein from duplicate samples was subjected to SDS PAGE followed by Western blot analysis using anti-GUS antibodies. The control lane is a sample

from leaves infiltrated with the CAMBIA 2300 (empty) vector. **c** Leaf extracts from the *uidA* infiltrated leaves were subjected to GUS enzyme activity determination using the fluorogenic substrate 4-MUG. The molar amount of the 4-methyl umbelliferone (4-MU) produced in the reaction was calculated against a 4-MU standard curve. The experiments were repeated three times with similar results

Fig. 5.

Analysis of in vitro translation products of in vitro produced transcripts corresponding to the different *Gmgl β 1* gene constructs (as described in Fig. 1). Total RNA was isolated from infiltrated tobacco leaves and reverse transcribed. GS₁ PCR products were further amplified from the cDNA with forward primers for the GS₁ 5'UTR (for constructs GS β 150 and GS β 153) or for the CaMV35S mRNA leader (constructs GS β 100 and GS β 103). RNA was in vitro transcribed from the RT-GS β ₁ clones and purified. Equal amounts of capped and polyadenylated RNA were translated in a wheat germ translation system and equal amounts of the translation reactions were analyzed by Western blotting with an anti-GS₁ antibody. The position of the GS β ₁ immunodetected bands is indicated by an *arrow*. The presence of an immunoreactive band in the wheat germ extract, that corresponds to the endogenous GS₁, is marked with an *asterisk*. GS β ₁ protein bands were quantified and the band intensity values corresponding to the GS constructs with or without its 5'UTR/3'UTR were plotted. The in

vitro transcription, translation experiments were repeated three times with the same results, only a representative Western blot is shown. *Double asterisk* Analysis showed that the differences in band intensities due to the presence of either the 5'UTR or the 3'UTR were not statistically significant ($P < 0.05$)

Fig. 6.

Secondary structure of the 5'UTR of the Gmgl β ₁ mRNA. The optimal secondary structure of the 5'UTR, including some nucleotides in the coding region (down to a BglII site), was predicted by MFOLD 2.0. The predicted free energy of $-27.1 \text{ kcal mol}^{-1}$ was calculated by the UNAFold 3.8 software. The translation start codon is labeled with an *asterisk*

Fig. 7.

Expression of the *Gmglnβ₁* gene in *Escherichia coli*. **a** Map of the constructs containing the *Gmglnβ₁* gene in the pGEX-4T-2 plasmid, showing the sequence of **b** the junction of the GST gene and the 5'UTR of the *Gmglnβ₁* gene in the plasmid pGEX::GS153 and **c** the junction of the GST and the CAMBIA mRNA leader in front of the *Gmglnβ₁* gene in the plasmid pGEX::GS103, with regards to sequences that may have a role in translation initiation in prokaryotes. SD represents Shine–Dalgarno-like sequences. **d** Expression of the plasmids containing the GST: *Gmglnβ₁* gene fusions in the pGEX-4T-2 plasmid. Expression was induced from the P_{tac} promoter with IPTG. Proteins were extracted after 2 h induction,

fractionated by electrophoresis, blotted and immunodetected using the antibodies against the soybean GS β ₁ protein, and against the GST protein. A sample from infiltrated tobacco leaves was included as control to indicate the migration pattern of the GS β ₁ protein in plants. A control sample from *E. coli* containing the pGEX-4T-2 plasmid was also included for comparison. An *asterisk* indicates the position of a nonspecific antigenic band in *E. coli*. *Lane numbers* represent samples from three independent single *E. coli* colonies